ROLE OF RECQ5 DNA HELICASE IN DNA DOUBLE-STRAND BREAK REPAIR BY HOMOLOGOUS RECOMBINATION

DISSERTATION

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SUMMARY

Double-strand breaks (DSBs), if left unrepaired or inaccurately repaired, can result in tumorigenesis or cell death. There are two major pathways for restoring genomic integrity at sites of DSBs; namely non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is highly error-prone and often results in deletions or chromosomal rearrangements. In contrast, HR in an error-free repair mechanism. HR reaction is initiated with extensive resection of the DSB ends resulting in formation of a 3'-ssDNA (single-stranded DNA) tail that is utilized by the RAD51 recombinase to generate a nucleoprotein filament. Such a filament is absolutely necessary for repair by HR pathway as it can perform the search for a homologous template and mediate strand invasion. Once the broken DNA end has been extended, further repair takes place *via* either of the two sub-pathways; one being synthesis-dependent strand annealing (SDSA), which has exclusively non-crossover (NCO) outcome, and the other being double-strand break repair (DSBR) pathway, which results in either NCO or crossover (CO) products. In somatic cells, repair by HR usually results in NCO outcome via the SDSA pathway. Inappropriate HR can be deleterious for the cell, for example COs between homologous chromosomes can lead to loss of heterozygosity (LOH) and COs between repetitive stretches of DNA can result in deletions or inversions. To avoid such outcomes, HR is subjected to regulation at several layers. The RecQ family of DNA helicases, comprised of RECQ1, BLM, WRN, RECQ4, and RECQ5 in humans, has evolved as caretakers of genomic integrity (Chu & Hickson, 2009).

The aim of this study was to gain mechanistic insight into the regulation of HR by RECQ5. It is established that loss of RECQ5 in mice results in genomic instability and a predisposition to cancer (Hu et al, 2007). Also, RECQ5 interacts physically with RAD51 and has the ability to disrupt RAD51-ssDNA filaments (Hu et al, 2007). The initial part of this PhD study focused on analysis of the mechanism used by RECQ5 to catalyze disruption of RAD51-ssDNA filaments. We could map the region on RECQ5 involved in its interaction with RAD51 and demonstrate that this region is partially responsible for RECQ5's ability to disrupt RAD51-ssDNA filaments. Furthermore, we could show that there exists an additional domain in RECQ5, between the Zinc-binding motif and the RAD51 interaction domain, which contributes to the filament disruption activity of RECQ5.

The second part of this PhD study dealt with the physiological relevance of RECQ5's ability to disrupt RAD51-ssDNA filaments. Using GFP-based reporters for different DSB repair pathways, we discovered that RECQ5 is required for HR with NCO outcome. Moreover, we found that RECQ5 counteracts the inhibitory effect of RAD51 on DSB repair by single-strand annealing (SSA), which is mechanistically similar to SDSA. Biochemical reconstitution of the annealing step during SSA and SDSA pathway further validated our cellular observation, suggesting that the presence of RAD51 is inhibitory during initiation of SDSA (and SSA) and that RECQ5 promotes annealing of the two DNA ends at the break site by relieving the inhibitory effect of RAD51. Final evidence for this hypothesis was provided by sister chromatid exchange (SCE) analysis, which suggested that RECQ5 and BLM act in separate pathways to suppress crossing-over during HR.

In view of our biochemical and cellular observations, we conclude that RECQ5 is required for HR. It is involved in suppression of mitotic crossing-over and promotes formation of non-crossover products by disrupting illegitimate RAD51-ssDNA filaments that might form during post-synaptic phase of SDSA.

ZUSAMMENFASSUNG

DNS-Doppelstrangbrüche (DSBs) können, falls nicht oder nur unzureichend repariert, zu Tumorbildung oder Zelltod führen. Es existieren zwei Hauptpfade, um die genomische Integrität bei Auftreten eines DSB wieder herzustellen; sie werden als Nicht-Homologes End-Joining (NHEJ) und Homologe Rekombination (HR) bezeichnet. Das NHEJ ist fehleranfällig und führt oft zu Deletionen oder chromosomalen Umlagerungen. Im Gegensatz dazu ist die HR ein fehlerfreier Mechanismus. Initiiert wird die HR durch umfangreiche Prozessierung der DSB-Enden, welche in 3'-Einzelstrang-DNS-Überhängen resultiert. Diese werden dann von der RAD51-Rekombinase verwendet, um Nukleoprotein-Filamente zu bilden. Solche Filamente sind unerlässlich für die HR, da sie die Suche nach einer homologen Sequenz ausführen und die sogenannte Strang-Invasion ermöglichen. Die weitere Reparatur der gebrochenen DNS-Enden erfolgt dann durch Synthese-abhängiges Strang-Annealing (SDSA), welches ausschliesslich zu nicht-Cross-over (NCO) Produkten führt, oder durch die klassische Doppelstrangbruch-Reparatur (DSBR), deren Folge entweder Cross-over (CO) oder NCO Events sind. In somatischen Zellen erfolgt eine Reparatur durch die HR in der Regel über den Weg des SDSA und erzeugt ein NCO Produkt. Fehlerbehaftete HR kann für eine Zelle schädlich sein, denn ein CO zwischen homologen Chromosomen könnte einen Verlust der Heterozygotie (LOH) nach sich ziehen, und CO zwischen repetitiven Sequenzen der DNS kann zu Deletionen oder Inversionen führen. Um solche Ereignisse zu verhindern, ist die HR auf mehreren Ebenen strengstens reguliert. Die RecQ-Familie der DNS-Helikasen bekleiden eine wichtige Rolle in der Aufrechterhaltung der genomischen Integrität und umfassen im Menschen die fünf Proteine RECQ1, BLM, WRN, RECQ4 und RECQ5 (Chu & Hickson, 2009).

Ziel dieser Arbeit war es, die Regulation der HR durch RECQ5 zu erforschen und den zugrunde liegenden Mechanismus aufzuklären. Von Studien mit Mäusen ist bekannt, dass ein Verlust von RECQ5 zu genomischer Instabilität führt und eine erhöhte Neigung zur Tumorbildung als Folge hat (Hu et al, 2007). Des Weiteren interagiert RECQ5 physisch mit RAD51 und hat die Fähigkeit, RAD51-Filamente aufzulösen (Hu et al, 2007). Die Analyse des zugrundeliegenden Mechanismus von RECQ5 um RAD51-ssDNS-Filamente zu destabilisieren, stand im Fokus des ersten Teils dieser Dissertation. Wir

konnten die Region, welche für die Interaktion zwischen RECQ5 und RAD51 verantwortlich ist identifizieren und zeigen, dass diese teilweise für die Fähigkeit zur Auflösung von RAD51-ssDNS-Filamenten durch RECQ5 verantwortlich ist. Des Weiteren wurde gezeigt, dass eine weitere Domäne in RECQ5, die zwischen dem Zn-Bindungsmotiv und der RAD51-Interaktionsdomäne liegt, an der Filament-Auflösungsfähigkeit von RECQ5 beteiligt ist.

Der zweite Teil dieser Arbeit befasste sich mit der physiologischen Bedeutung der Fähigkeit von RECQ5 um RAD51-Filamente aufzulösen. Durch die Verwendung von verschiedenen GFP-basierenden Reportersystemen, die jeweils einen der Reparaturmechanismen von DSBs repräsentieren, wurde ermittelt, dass RECQ5 in der HR beteiligt ist die zu einem NCO-Ergebnis führt. Weiterhin haben wir herausgefunden, dass RECQ5 dem inhibitorischen Effekt von RAD51 in der DSB-Reparatur durch den Einzelstrang Annealing-Pfad (SSA) entgegenwirkt, welcher mechanistisch sehr ähnlich zum SDSA ist. Die biochemische Rekonstruktion des Annealing-Schrittes des SSA und des SDSA-Pfades bestätigte die zuvor in Zellen gemachten Beobachtungen und deutet darauf hin, dass die Anwesenheit von RAD51 hemmend auf die Initiierung des SDSA (und des SSA) wirkt und dass RECQ5 das Aneinanderlagern der beiden DNS-Enden an der Bruchstelle bewirkt, indem der inhibitorische Effekt von RAD51 aufgehoben wird. Weitere Beweise dafür konnten in einer Schwesterchromatid-Austausch-Analyse gefunden werden, die darauf hindeutete, dass RECQ5 und BLM in separaten Mechanismen fungieren um Crossing-over während der HR zu unterdrücken.

Aus unseren biochemischen und zellulären Beobachtungen kann geschlossen werden, dass RECQ5 für die HR erforderlich ist. Dieses Protein ist in der Unterdrückung von mitotischen Cross-over Events involviert und fördert die Bildung von nicht-Cross-over Produkten, indem unzulässige RAD51-ssDNS-Filamente aufgelöst werden, die in einer späteren Phase des SDSA gebildet werden könnten.

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ABBREVIATIONS

Double-strand breaks	DSBs
Non-homologous end joining	NHEJ
Homologous recombination	HR
Synthesis-dependent strand annealing	SDSA
Double-strand break repair	DSBR
Non-crossover	NCO
Crossover	CO
Loss of heterozygosity	LOH
Single-stand annealing	SSA
Sister chromatid exchange	SCE
Genetic instability	GIN
Nucleotides	ntds
Chromosomal instability	CIN
DNA damage response	DDR
Immunoglobulin	Ig
Reactive oxygen species	ROS
Single-strand breaks	SSBs
Intra- and inter-strand crosslinks	ICLs
Fanconi anemia	FA
DNA-dependent protein kinase catalytic subunit	DNAPKcs
Ionizing radiation	IR
Displacement loop	D-loop
Replication protein A	RPA
ATR-interacting protein	ATRIP
cyclin-dependent kinases	CDKs
Double Hollliday junction	dHJ
Break-induced replication	BIR
Structure-specific endonucleases	SSEs
Homology-directed repair	HDR
Mouse embryonic stem	mES

Mouse embryonic fibroblasts	MEFs
Adenosine triphosphate	ATP
Serine/threonine	Ser/thr
Super-families	SF
Caenorhabditis elegans	*C. elegans*
Escherichia coli	*E. coli*
Rothmund-Thomson syndrome	RTS
Baller-Gerold syndrome	BGS
RQC	RecQ C-terminal
HRDC	Helicase and RNaseD C-terminal
Winged-helix	WH
Nuclear localization signals	NLS
Holliday junctions	HJs
Saccharomyces cerevisiae	*S. cerevisiae*
Topoisomerase III	top3
Methylmethane sulfonate	MMS
Ultra-violet	UV
Flap-endonuclease 1	FEN1
Amino acid	aa
Doxycycline	Dox
Phosphate-buffered saline	PBS
Fetal calf serum	FCS

1 INTRODUCTION

1.1 Genome stability and cancer

The term genome instability (or genetic instability, GIN) refers to the entire repertoire of genetic alterations occurring in a cell due to many different reasons. The spectrum of these alterations ranges from point mutations, small insertions or deletions of nucleotides (ntds) to gross chromosomal rearrangements (Myung et al) such as translocations, duplications, inversions or deletions. They also include a condition called Aneuploidy where the cell has an extra or missing chromosome (Draviam et al, 2004). Changes in chromosome number and structure are also referred to as Chromosomal instability (Gonzalez-Suarez et al) (Gonzalez-Suarez et al). Genomic instability is a characteristic of all cancers with CIN being the major form of instability. Furthermore, GIN is fuelled by DNA damage, errors during replication or defective/inaccurate DNA repair pathways.

More than a decade ago, six hallmarks of cancer were described that were used to define the functional capabilities of a cancerous cell. This included: self sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis, and unlimited replication potential (Hanahan & Weinberg, 2000). At that time, GIN was considered to be a property of cancerous cells that served them to attain the six hallmarks. But now additional hallmarks that describe the state of cancer cells have been included, namely: DNA damage and DNA replication stress, oxidative stress, mitotic stress, proteotoxic stress, metabolic stress and GIN (Kroemer & Pouyssegur, 2008; Luo et al, 2009; Negrini et al, 2010). The inclusion of GIN as one of the hallmarks of cancer stems from the observation that it is present in all stages of cancer (Lengauer et al, 1997).

The mutator hypothesis establishes the idea that precancerous lesions have GIN due to mutations in caretaker genes (such as DNA repair genes and mitotic checkpoint genes) and this helps them drive tumorigenesis by enhancing the accumulation of spontaneous mutations (Loeb, 1991). This is exemplified by hereditary cancers, which show CIN/GIN and are driven by mutations in DNA repair genes. On the other hand, although sporadic/non-hereditary cancers show GIN and mutations in a large number of

genes, they very often do not have mutations in the caretaker genes. GIN in such cancers can be explained by the oncogene-induced DNA replication stress model (Halazonetis et al, 2008). This phenomenon leads to collapse of replication forks, especially at common fragile sites, followed by the formation of DNA double-strand breaks and activation of the DNA damage response (DDR) pathway. Breaks at common fragile sites can drive GIN and a constitutively activated DDR also leads to elevated levels of errors during DNA repair, favoring the transition of a precancerous lesion into cancer (Bartkova et al, 2005; Gorgoulis et al, 2005).

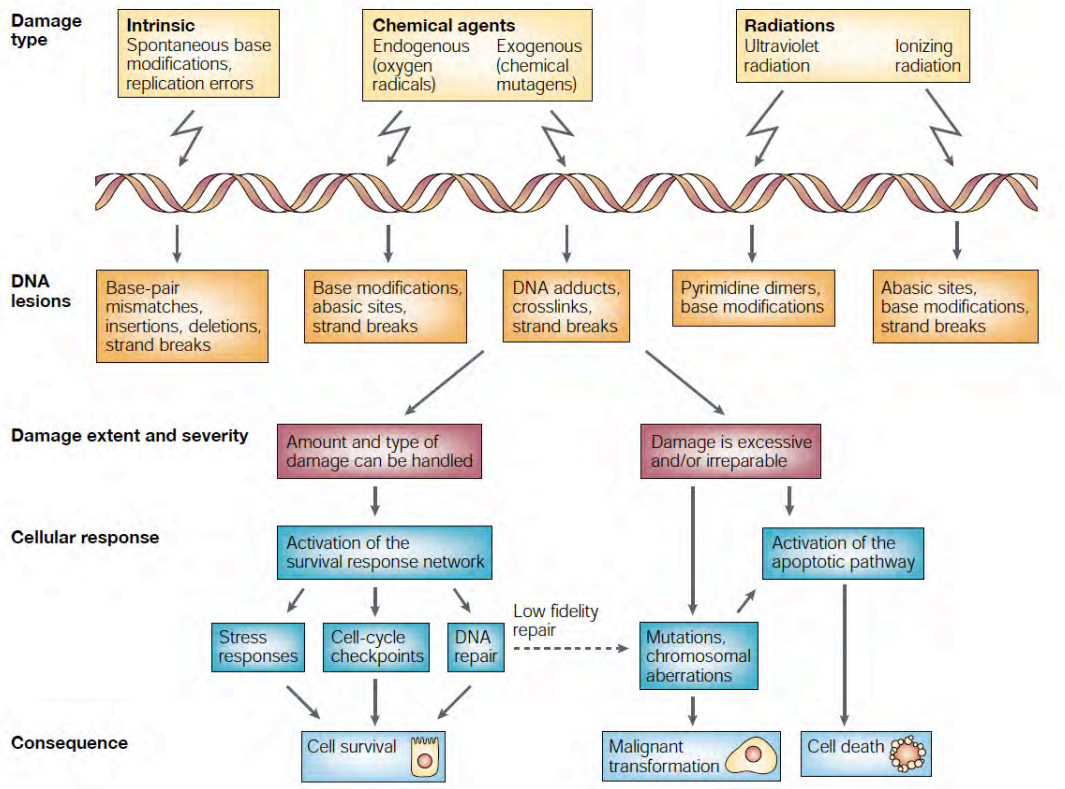


Figure 1: **DNA damage and its effect at the cellular level.** Over the course of evolution, the cell has developed various mechanisms to deal with all kinds of DNA lesions resulting from various endogenous or exogenous insults. The outcome from such pathways could be accurate repair, introduction or loss of genetic information, or cell death. Accumulation of genetic mutations can eventually lead to transformation of the cell into a cancerous cell. Figure adapted from Shiloh, 2003 (Shiloh, 2003).

The DNA damage response is closely associated with other human diseases as well. Mutations in DDR genes often affect the balance in the physiology of nervous, immune, and reproductive systems. Also, such mutations have a marked role in premature

aging and increased predisposition to cancer (Jackson & Bartek, 2009). Although DNA damage and mutations, if not controlled can drive pathological disorders; they are also essential at the same time to create genetic variations that push evolution. For example, immunoglobulin (Ig) diversification relies on processes such as somatic hypermutation and class switch recombination that are essential for normal development of the immune system (Maizels, 2005). Meiotic cell division relies on the induction of DSBs by the Spo11 nuclease leading to formation of COs, between the homologous chromosomes, that are essential for proper chromosome segregation (Keeney & Neale, 2006).

1.2 DNA damage and repair

1.2.1 DNA damage: Need and Sources

Although, for purposes of evolution, it is good to maintain genetic variability through acquisition of mutations in the germline DNA, changes in the genetic material of somatic cells are usually unwanted as even one mutation can set the platform for onset of malignancy. Cells have developed a range of mechanisms to safeguard or/and rectify such changes and maintain genetic stability (Thompson & Schild, 2002). These mechanisms are operative at all times due to the high incidence of DNA damage resulting from common factors such as heat, oxidative damage, hydrolytic attack, reactive oxygen species (ROS), spontaneous base loss, environmental chemicals, ultraviolet radiation, and ionizing radiation (Rouse & Jackson, 2002; Schar, 2001).

The innumerable DNA lesions that the cell accumulates every day need to be repaired efficiently because, if left unrepaired, they pose a threat to faithful transmission of genetic material. Damage to DNA can be due to either endogenous or exogenous sources, but the types of DNA damage produced are overlapping. Endogenous sources include deoxyribonucleotide triphosphate misincorporation during replication resulting in mismatches. Though such errors happen once every 10^3 to 10^4 bases replicated, they can be fatal as they can result in a change of the coding property of the DNA molecule. Stalled replication forks at sites of DNA damage can result in DSBs. ROS can lead to modification of bases or the sugar-phosphate backbone by oxidation. Spontaneous metabolic events can also lead to alkylation, deamination, complete loss of base (depurination or

depyrimidination), or to addition of bulky chemical adducts (Lindahl & Barnes, 2000). Exogenous sources include environmental factors such as heat, cigarette smoke, sunlight etc. Exposure of cells to ultra-violet (UV) light causes dimerization of pyrimidines and 6-4 photoproducts. Ionizing radiation (IR), used in medical imaging and cancer therapy, also results in a variety of lesions such as DNA base damage, intra- and inter-strand cross links (ICLs), single-strand breaks (SSBs), DSBs, and DNA-protein cross links (Hoeijmakers, 2009; Jeggo & Lobrich, 2006). DSBs can also arise from chemical agents such as DNA topoisomerase I and II inhibitors (e.g. camptothecin, topotecan, etoposide and doxorubicin), which block the re-ligation activity of the topoisomerase (Banath & Olive, 2003; Hammond et al, 2003), as well as from radio-mimetic drugs such as bleomycin and neocarzinostatin which work via the production of ROS (Banath & Olive, 2003; Lobrich & Jeggo, 2007; Pommier et al, 2010).

As mentioned previously, DNA damage is not always unwanted. In special conditions, DSBs are purposefully created. For example, RAG proteins create DSB during V(D)J recombination that gives rise to the entire repertoire of antibodies (Fugmann et al, 2000; Jung et al, 2006).

1.2.2 Pathways to repair DNA damage

In eukaryotes, DNA repair pathways can be classified into five major categories; namely (1) direct repair, (2) base excision repair (BER), (3) nucleotide excision repair (NER), mismatch repair (MMR), and (5) DSB repair.

Direct repair pathway is a single step-single enzyme pathway, which can be further divided into two sub-pathways: alkylation damage repair and UV-induced photoproduct repair. In humans, the enzyme O^6-methylguanine-DNA methyltransferase (MGMT) repairs alkylation damage by transferring the methyl group from O^6-methylated guanine to a cysteine residue in its active site. Another class of enzymes called photolyases removes UV lesions.

The basic mechanism for BER, NER, and MMR is similar and involves excision of the DNA lesion from the affected strand, synthesis of the missing sequence using the intact opposite strand by polymerases and ligation of the nicks by DNA ligases. BER functions to correct abasic (AP) sites and modified bases caused by oxidation, alkylation, deamination. DNA glycosylases are a group of enzymes that recognize the modified bases

and remove them by cleavage of the N-glycosidic bond. The resulting AP site is processed by an AP endonuclease followed by recruitment of the polymerase and ligases. NER machinery works by recognizing unusual distortions in the DNA helix rather than specific lesions. Such distortions are usually a result of either bulky adducts or UV-induced crosslinking of adjacent pyrimidine bases. Errors incorporated during replication are often missed by the proofreading activity of the polymerases. Such base mismatches and small insertion/deletion loops are repaired by the MMR pathway. It is initiated by binding of MutSα (MSH2-MSH6) or MutSβ (MSH2-MSH3) to the lesion followed by recruitment of MutLα (MLh1-PMS2). This complex then slides along the DNA in search of a nick. The DNA strand containing the nick is then processed until the mismatch has been removed followed by gap filling and ligation.

The DSB repair pathways are described in detail in the following section(s). SSBs are repaired following detection by PARP1 [Poly (ADP-ribose) polymerase 1] and recruitment of factors involved in 'cleaning' the ends, gap-filling and ligation. If such SSBs are encountered by the replication machinery, they are converted into DSBs that can then be repaired by the DSB repair pathways. Another class of DNA damage includes ICLs. Repair of such lesions is mediated by the Fanconi anemia (FA) proteins and the HR pathway.

1.3 Double-strand break repair pathway

1.3.1 Non-homologous end joining

The least complicated way of repairing a DSB is direct re-ligation of the broken ends without paying much attention to the genetic sequence at the break (Weterings & van Gent, 2004). This process is called non-homologous end joining (NHEJ) and it results in accurate repair for simple, clean breaks like blunt ends (van Heemst et al, 2004). But when the ends are not compatible, like those induced by IR and radio-mimetic drugs, due to the presence of non-ligatable, chemically modified nucleotides, they need to be processed before ligation and this results in sequence alteration at the break site. This loss of nucleotides from the site of damage makes NHEJ an error-prone process.

NHEJ is the most important DSB repair pathway in cells and is operative throughout the cell cycle (Branzei & Foiani, 2008; Rothkamm et al, 2003). Contrary to what the name suggests, NHEJ also requires 1-6 bp region of homology at the break site. As soon as a DSB is created in the cell, it is targeted for repair *via* NHEJ by rapid binding of the heterodimeric Ku70/80 complex to each end at the break site (Gottlieb & Jackson, 1993; Mimori & Hardin, 1986). This heterodimer has no sequence specificity for binding to DNA. Instead it forms a ring-like structure that loads onto the broken DNA end and diffuses away from the break site (de Vries et al, 1989). The binding of Ku70/80 helps to stabilize the two DNA ends and bring them closer together for ligation. This is particularly useful when the ends are blunt and there is no homology to facilitate base pairing (Ramsden & Gellert, 1998). Also, it serves as a platform to recruit rest of the NHEJ machinery beginning with the DNA-dependent protein kinase catalytic subunit (DNAPKcs) (Lieber, 2010). Lastly, Ku70/80 binding also helps to prevent 5' resection of the break ends, which is a prerequisite for HR. The complex of Ku70/80 heterodimer with DNA and DNAPKcs is referred to as the DNAPK holoenzyme. The binding of DNAPKcs to DNA and the Ku70/80 complex activates its serine/threonine (Ser/thr) kinase activity resulting in its auto-phosphorylation and phosphorylation of the H2AX histone variant (Stiff et al, 2004). This is followed by the recruitment of the MRE11-RAD50-NBS1 (MRN) complex and although its exo- and endonuclease activities are dispensable for NHEJ, the zinc hook domains of RAD50 helps Ku70/80 and DNAPKcs in tethering the broken DNA ends together (Hopfner et al, 2002). The ends are then ligated by the ligase IV in complex with XRCC4 and XLF (XRCC4-like factor), the latter two being required for efficient targeting of the DNA ligase IV to the broken DNA ends and stimulation of its ligase activity (Ahnesorg et al, 2006; Mari et al, 2006; Nick McElhinny et al, 2000).

Many times, the break ends are incompatible and need to be processed prior to ligation. For example, IR-induced DSBs contain 5'-hydroxyl and 3'-phosphate groups that cannot be ligated together without processing. In mammals, such ends are corrected by the recruitment of a polynucleotide kinase (PNK) to the break site by XRCC4 as it has both a 5'-kinase and a 3'-phosphatase activity (Chappell et al, 2002; Koch et al, 2004). XRCC4 interacts with another protein, Aprataxin, which can remove adenylate groups from 5'-phosphates at the break site, resulting in a ligatable end (Ahel et al, 2006; Clements et al, 2004). Sometimes, the DNA ends have incompatible overhangs or special structures like

hairpin, for example during V(D)J recombination, and this requires nucleolytic processing of the ends. In mammals, a nuclease called Artemis is recruited to the break ends and is activated upon its phosphorylation by DNAPKcs. Artemis can endonucleolytically process 3'- and 5'-overhangs and open hair-pin structures generated during V(D)J recombination (Ma et al, 2002). It is suggested that WRN helicase/exonuclease could also be involved in the exonucleolytic processing of DNA ends upon stimulation by Ku70/80 heterodimer (Perry et al, 2006). Lastly, incompatible ends can also be filled-in by X family DNA polymerases such as polμ and polλ (Nick McElhinny et al, 2005).

To sum up, NHEJ is the simplest and fastest way to repair a DSB in all phases of the cell cycle. Loss of NHEJ pathway leads to severe immunodeficiency in mammals. However, due to its propensity to incorporate errors, NHEJ can lead to genomic instability.

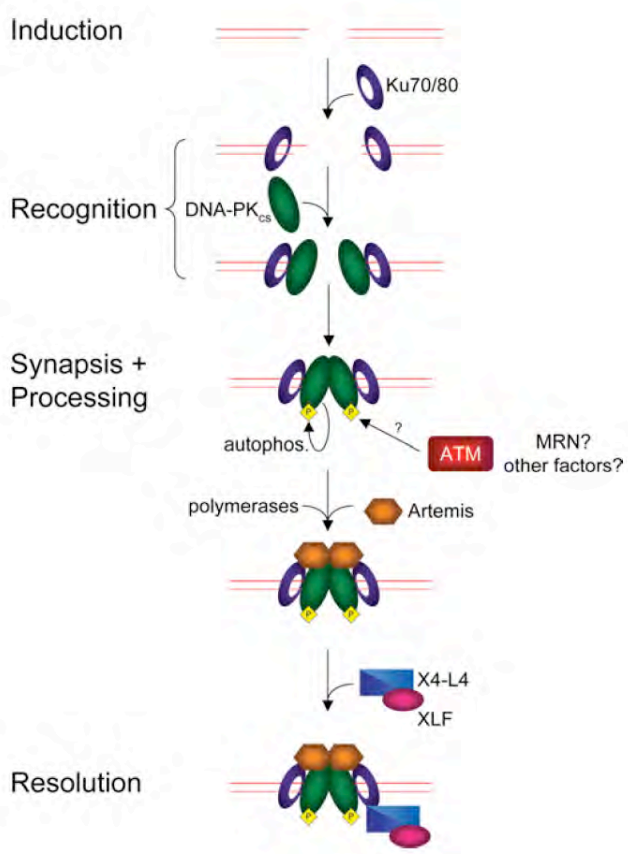


Figure 2: **Mechanism of NHEJ.** Upon induction of a DSB, DNA ends are bound by the Ku70/80 heterodimer. This is followed by recruitment of DNAPKcs to each DNA end and formation of a DNAPK holoenzyme. This recognition step is followed by synapsis and processing, wherein the end are tethered together and the phosphorylation activity of DNAPKcs is activated. Beginning with self-phosphorylation, DNAPKcs undergoes a conformational change and recruits other NHEJ repair

factors like artemis, polμ and polλ which process the DNA ends. Finally, XRCC4-XLF-LigaseIV complex repairs the break by ligating the ends. Figure adapted from Hartlerode and Scully, 2009 (Hartlerode & Scully, 2009).

1.3.2 Alternative end joining

Research in the last few years has led to the discovery of a Ku-independent repair pathway operative throughout the cell cycle. Although this pathway was initially considered to be a 'back-up' repair pathway in NHEJ deficient cells, recent studies have shown that it is functional in NHEJ proficient cells as well (Corneo et al, 2007). This pathway is called alternative non-homologous end joining (alt-NHEJ) or microhomology-mediated end joining (MMEJ) and is particularly useful when the DSB ends are incompatible and require processing (McVey & Lee, 2008). For example, treatment of cells with camptothecin results in inhibition of topoisomerase I (top I), which is left covalently attached to the break end. Such a DNA end cannot be bound by Ku70/80 heterodimer, thus preventing NHEJ initiation. This scenario requires nucleolytic processing of the blocked DNA end and resection of one strand of the break so that small regions (5-20 bp) of homology can be uncovered. The ends are then aligned together and stabilized by base pairing between the complementary sequences, followed by removal of the displaced DNA flap by nucleases and ligation. The removal of overhangs implies that the DNA sequences flanking the break site are removed, resulting in a deletion. MMEJ seems to harness its machinery from both NHEJ and HR repair pathways. The processing and resection of broken ends requires MRE11, EXO1 and CtIP (Lee & Lee, 2007; Ma et al, 2003). Although CtIP is conventionally considered to be a promoter of HR, it shows limited resection activity during G1. Upon its phosphorylation at Ser327, during G1/S transition, it recruits BRCA1 to the DSB site and shifts the balance in favor of HR (Yun & Hiom, 2009). Once the complementary sequences have been annealed together, the non-complementary 3'-flaps are removed from the annealed substrate by the structure-specific endonuclease XPF-ERCC1 (Rad1-Rad10 complex in yeast) (Ahmad et al, 2008). Finally, gap filling is followed by ligation mediated by either Ligase I or Ligase IIIα in a complex with XRCC1 (Caldecott et al, 1994; Liang et al, 2008).

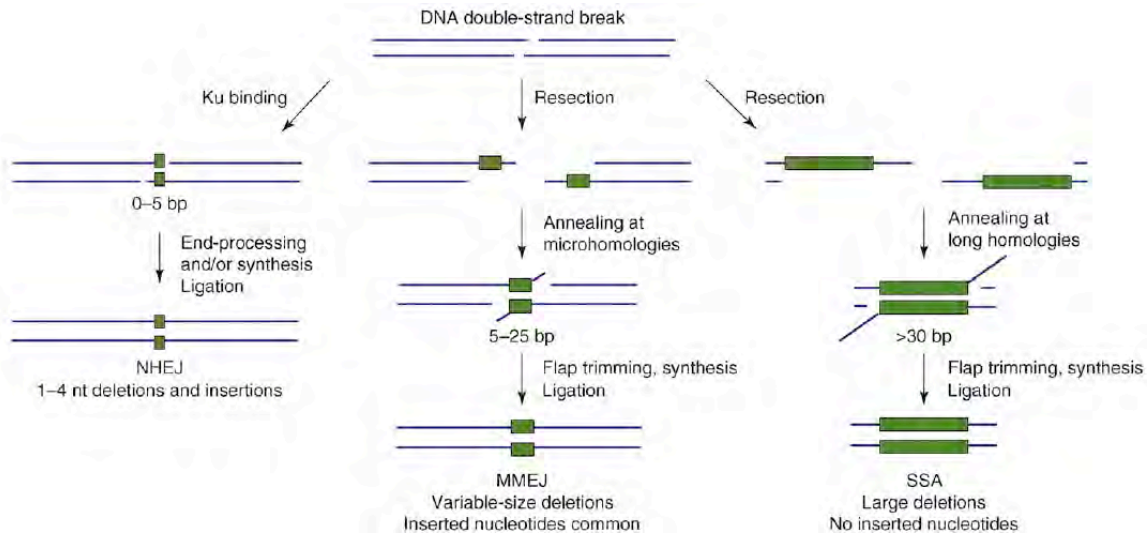


Figure 3: **Different end joining pathways**. Although the three pathways look different, they have a similar mechanism and overlapping genetic requirements. Repair of a DSB by NHEJ requires Ku70/80 binding to the break ends and this prevents resection. This is followed by annealing between 0-5 bp stretch of short homologies, filling-in by polymerases and ligation. This sometimes results in insertions or deletions at the break site. The other two end joining pathways, MMEJ and SSA, require DNA end resection until homologous regions are found which is then followed by 3'-flap cleavage, filling-in and ligation. MMEJ requires 5-25 bp of homology and SSA involves >30 bp of homology. Both MMEJ and SSA are always mutagenic. Figure adapted from McVey and Lee, 2008 (McVey & Lee, 2008).

Single-strand annealing (SSA) pathway is also a type of alternative end joining pathway and is initiated by resection of the DSB end. If resection reveals more than 30 bp long repeated sequences in the same orientation, in the DSB flanking DNA tails, then repair can take place by SSA pathway. Conceptually, the process is similar to MMEJ but SSA requires different genetic factors. Once the complementary sequences have been revealed by extensive 5' to 3' resection, the two 3'-DNA tails are aligned together and annealed in the presence of RPA and Rad52 (Prado & Aguilera, 1995; Sugawara et al, 2000; Van Dyck et al, 2001). This is followed by removal of overhanging flaps by Rad1-Rad10 along with Msh2-Msh3 and Slx4, filling-in by DNA polymerases and ligation (Fishman-Lobell & Haber, 1992; Flott et al, 2007; Saparbaev et al, 1996). Other factors influencing SSA include RAD51. Although absolutely mandatory for HR, it was seen that over-expression of RAD51 inhibits SSA (Stark et al, 2004). This makes sense because there is no requirement of displacement loop (D-loop) formation during SSA. Also, it was

shown biochemically for yeast Rad51 that it inhibits Rad52 and RPA mediated annealing of DNA strands (Wu et al, 2008). SSA is a mutagenic way of repairing DSBs and always results in deletion of sequences between the repeats and one of the repeats itself. Nevertheless, such a pathway could be involved in the repair of tandem repeats found in the rDNA locus. SSA might also help the cell survive under conditions of excessive DNA damage or in case of an HR defect.

1.3.3 Homologous recombination

Homologous recombination (HR), also known as homology directed repair (HDR), takes place predominantly during S phase of the cell cycle when replication is taking place and a sister chromatid is available to serve as a template for accurate repair of the broken DNA end. As soon as a DSB is detected, the MRN complex is recruited to the break ends (Hopfner et al, 2002; Williams et al, 2009) and along with the cohesin complex SMC5/6 helps in stabilizing the DNA ends and holding them together in close proximity (Feeney et al, 2010; Williams et al, 2007). MRN complex is also involved in recruitment and activation of the ATM protein kinase (Uziel et al, 2003; Williams et al, 2007). ATM is involved in phosphorylation of various DDR players including the C-terminal tail of the histone variant H2AX (γH2AX). This histone phosphorylation marks the site of the DSB lesion, but exact implication of this modification is still unknown. Such a 'marked' lesion is then recognized by MDC1 (mediator of DNA damage checkpoint 1), which binds to γH2AX and recruits additional MRN through binding to phosphorylated NBS1 (Stucki et al, 2005). This triggers recruitment of the downstream players in the DDR signaling and repair pathway.

Two of the important downstream proteins are 53BP1 and BRCA1 because their recruitment decides whether the DSB will be repaired *via* NHEJ or HR. This is also regulated partly by MDC1 through its interaction with RNF8, an ubiquitin ligase. Recruitment of RNF8 initiates ubiquitylation of histones H2A and H2AX (Huen et al, 2007; Mailand et al, 2007) and this is propagated along the chromatin by RNF168, another ubiquitin ligase (Doil et al, 2009; Stewart et al, 2009). DSB repair proteins with ubiquitin interaction motifs (UIMs) get recruited to this ubiquitin platform. BRCA1 gets recruited to the DSB end as part of a complex that contains proteins with UIMs such as RAP80 and

this tilts the balance for DSB repair in favor of HR (Kim et al, 2007; Sobhian et al, 2007; Wu et al, 2009).

NBS1 subunit of the MRN complex also recruits CtIP and together they initiate 5'-3' DSB end resection (D'Amours & Jackson, 2002; Sartori et al, 2007). Immediately after this, BLM together with DNA2 and EXO1 leads a wave of extensive resection (Gravel et al, 2008; Nimonkar et al, 2011). Mre11 further speeds up the process of resection by initiating a bi-directional resection activity (Garcia et al, 2011). Probably what differentiates Alt-EJ and HR is the recruitment of Exo1 during HR, which leads to extensive resection of the break end (Mimitou & Symington, 2008). In prokaryotes like *Escherichia coli* (*E. coli)*, the RecBCD complex performs this step but in eukaryotes many different helicases and nucleases join together.

The resulting 3'-ssDNA tails can be several kilobases (Kb) in length and are bound and protected from degradation, by other exo- or endonucleases, by the heterotrimeric ssDNA binding protein, RPA (Replication protein A). This RPA-DNA assembly interacts with ATRIP (ATR-interacting protein) which is in complex with ATR (Zou & Elledge, 2003). Binding of ATR-ATRIP complex to DNA activates ATR through TOPBP1 interaction (which in itself gets activated by ATM) and this results in CHK1 phosphorylation and subsequent activation. This in turn leads to CHK1-mediated phosphorylation of proteins that help to spread the DDR throughout the nucleus. One example is CDC25, a protein phosphatase that regulates cell cycle transition by removing inhibitory phosphorylation of cyclin-dependent kinases (CDKs). Upon phosphorylation, CDC25 can no longer activate CDKs and the cell is prevented from entering mitosis (Cimprich & Cortez, 2008; Furnari et al, 1997). ATR also targets proteins in the HR pathway like BLM, WRN etc. and those at the replication fork like MCM2, RPA, various polymerases etc (Cimprich & Cortez, 2008).

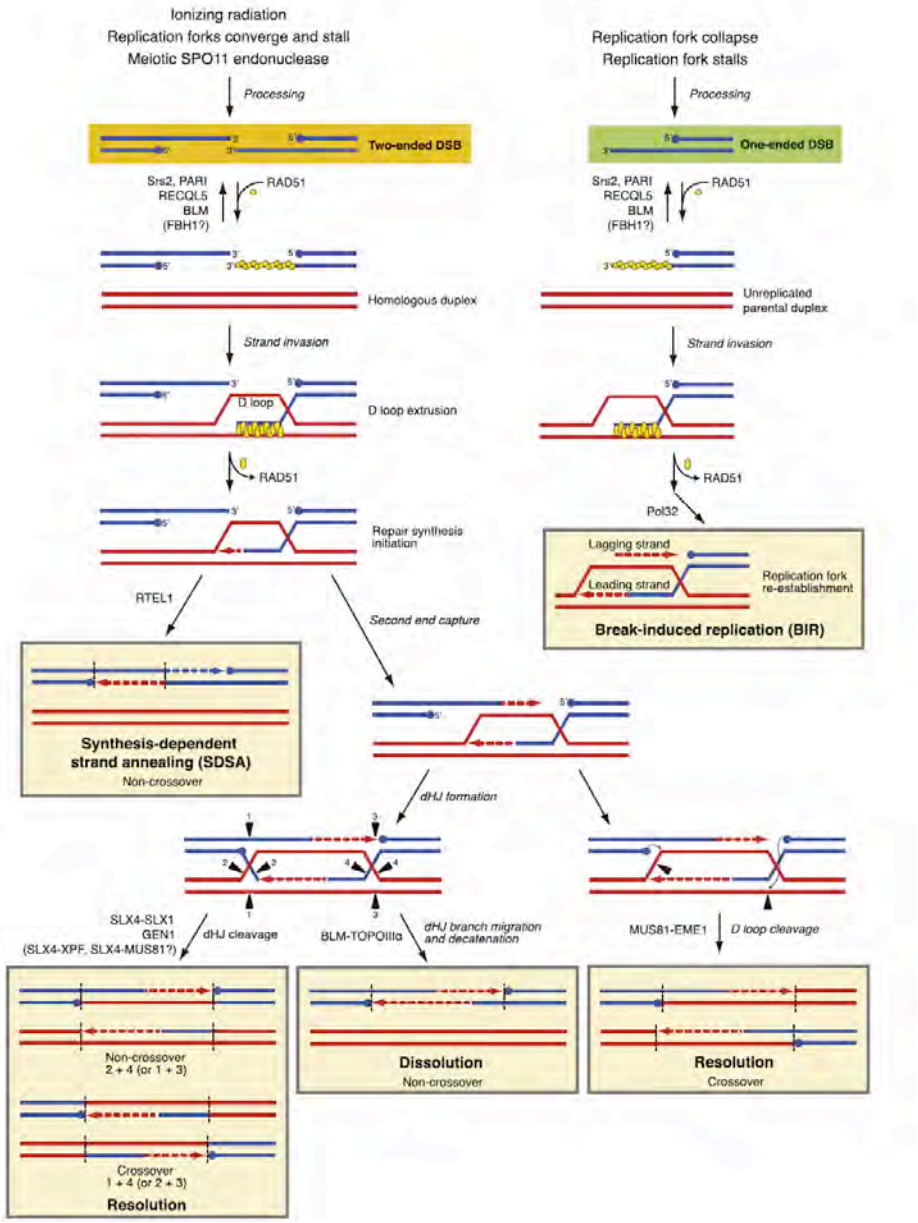


Figure 4: **Mechanism of homologous recombination**. Homologous recombination (HR) can initiate from either classical DSBs or from one-ended DSBs arising during replication. In both cases, mechanism of HR and the outcome remain the same. Commitment to HR takes place upon formation of the RAD51 presynaptic filament, which then invades the homologous duplex. Extension of the broken DNA end is followed by either second-end capture to promote dHJ formation or D-loop disruption to promote SDSA. In case of BIR, repair proceeds *via* re-establishment of the replication fork. In mitotic cells, HR usually results in non-crossover products. Figure adapted from Chapman and Boulton, 2012 (Chapman et al, 2012b)

At this step, if the 3'-ssDNA tails contain highly repetitive DNA sequences like Alu elements then SSA can initiate and repair the lesion resulting in deletion at the break

site. If the repair proceeds via HR then RPA is displaced from the 3'-ssDNA tail by RAD51 with the assistance of mediator proteins to form a RAD51 presynaptic filament. There are three different types of mediators but their mechanism of action is not yet understood. One group of mediators includes RAD51 paralogs: four proteins (Rad55-Rad57, Shu1-Psy3) in yeast and five proteins (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3) in mammals. These proteins are similar to RAD51 due to presence of the RecA core but they cannot form filaments on ssDNA and are unable to catalyze strand invasion or strand exchange reactions. The second mediator, best studied in yeast, is the Rad52 protein and it helps in loading Rad51 onto the ssDNA. The third mediator protein type is represented by BRCA1/BARD1, BRCA2 (also known as FANCD1), and PALB2 (also known as FANCN) (Sy et al, 2009; Zhang et al, 2009a). BRCA2 probably functions through its ssDNA binding sites, dsDNA binding and RAD51 binding sites. In humans, BRCA2 seems to have taken over the function of the yeast Rad52 during presynaptic filament formation.

A class of proteins called antirecombinases can disrupt the RAD51 filament. They are important to prevent untimely/illegitimate HR events. In yeast, Srs2 is the archetype antirecombinase but in mammals there are many potential candidates like FBH1, PARI, BLM and RECQ5. The recombinogenic presynaptic filament commits the cell to perform repair *via* HR and in the next step, RAD51 filament carries out search for a homologous template. This is followed by strand invasion of the template duplex resulting in the formation of a D-loop. The 3'-end of the presynaptic filament, or the invading strand, can prime DNA synthesis in the presence of DNA polymerases like polη using the homologous partner as template (McIlwraith et al, 2005). Another protein involved at this step is the RAD54 ATPase. It is involved in stabilizing the RAD51-ssDNA filament and promoting D-loop formation. It also promotes DNA synthesis by disrupting the RAD51 assembly from the heteroduplex DNA (Heyer et al, 2006). The latter function can also be mediated by HELQ-1 (Ward et al, 2010).

Once the 3'-end has been extended, there are three sub-pathways of HR that can take place namely, (1) synthesis-dependent strand annealing (SDSA) pathway, (2) double Hollliday junction (dHJ) or classical DSBR pathway, and (3) break-induced replication (Deckbar et al).

In the SDSA pathway, antirecombinases like RTEL1 dissociate the D-loop and the extended invading strand anneals with the other resected DNA end at the break site in presence of Rad52 and RPA (Van Dyck et al, 2001; Wu et al, 2008). If the 3'-flaps are formed, they are nucleolytically processed by structure-specific endonucleases (SSEs) like XPF/ERCC1 (Adair et al, 2000; Niedernhofer et al, 2004). The gaps, if any, are filled in by polymerases like polδ or polε and the ends are ligated by the DNA ligase I. This pathway always results in the formation of non-crossover (NCO) products with short gene conversion tracts. Thus, RTEL1 promotes SDSA and helps limit formation of COs in mitotic and meiotic cells (Youds et al, 2010). Recent studies have shown that mitotic cells almost always employ the SDSA sub-pathway during HR repair (Mitchel et al, 2010).

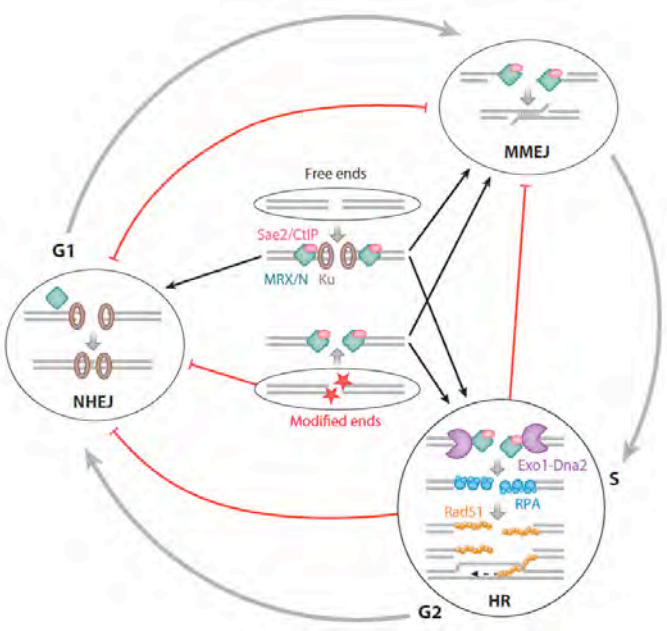
In the classical DSBR pathway, the second end at the break site is captured by the non-template strand in the D-loop structure resulting in the formation of a dHJ. This is followed by either dissolution of dHJ, which involves branch migration of the dHJ away from the break site sequence followed by topological decatenation of the dHJ structure by concerted action of BLM, TOPO IIIα and BLAP75 resulting in NCO products (Luo et al, 2000; Raynard et al, 2006; Wu & Hickson, 2003). The dHJ structure can also be resolved by various SSEs like GEN1, which nicks the HJ structure symmetrically one nucleotide away from the junction and resulting in ligatable products with no flaps (Ip et al, 2008). The SLX1-SLX4 complex also cleaves symmetrically but two nucleotides away from the junction (Svendsen & Harper, 2010). As SLX4 is found in complex with SLX1, MUS81, and XPF, it seems that SLX4 functions as a structural scaffold through which various SSEs can act. The Mus81-Eme1 complex cleaves HJs which have been already been nicked (Osman et al, 2003). No matter which complex is utilized for resolution, it results in the formation of either NCO or CO products. However, recent evidence suggests that resolution of dHJ is constrained towards CO formation (Mitchel et al, 2010).

Finally, there exists a modified version of HR called BIR, which is operative only at telomeres or broken replication forks (Strumberg et al, 2000). It is initiated just like HR beginning with extensive resection, formation of a presynaptic filament, homology search and strand-invasion followed by extension of the 3'-end. Unlike classical HR, here the extension requires re-establishment of the entire replication apparatus including pol32 subunit of polδ (Lydeard et al, 2007) and the extension of the 3'-end continues till the end of the chromosome template. At the same time, the other end from the break site is also

captured by the non-template strand of the D-loop, resulting in formation of a HJ. The other strand is also extended similarly till the end and then the HJ is resolved by MUS81-EME1 (Chen et al, 2001; Ciccia et al, 2003).

1.3.3.1 Pathway choice during DSB repair

Although a multitude of DNA DSB repair pathways have been developed by the cell, not all of these are competing with each other to repair a DSB. Each pathway is subject to various levels of regulation. The first level of regulation is dependent on the structure of DNA ends (Fig. 5). DSBs produced in the cell can be either 'clean' or 'dirty'. The DNA ends produced by restriction endonucleases are considered to be 'clean' as they have a 3'-hydroxyl and 5'-phosphate group and can be simply ligated together by NHEJ without further processing. Ends produced by IR or DSBs which have a protein covalently bound to the 5'-end are considered to be complex or 'dirty' and require initial processing like nuclease trimming or resection. The next level of regulation is at the stage of cell cycle. The factors like absence of sister chromatid and a highly compacted chromatin promote



NHEJ during G1.

Figure 5. **Regulation of pathway choice during DSB repair**. Free or clean DNA ends can be repaired by NHEJ, MMEJ or HDR. Unclean or damaged ends can be processed by either MMEJ or HDR but require processing before they can undergo NHEJ. MMEJ and NHEJ operate throughout

the cell cycle but HDR (HR and SSA) takes place only during S and G2 phases due to the requirement for resection of DNA ends. Figure adapted from Symington and Gautier, 2011 (Symington & Gautier, 2011)

Another important layer of regulation comes from the action of factors like 53BP1 and BRCA1. Both tumor suppressors are enriched at sites of DSBs and are important regulators of pathway choice between NHEJ and HR. The role of 53BP1 and BRCA1 in pathway choice is very specific and is independent of other core proteins of NHEJ and HR pathway. Cells deficient for BRCA2 or XRCC2 cannot be rescued by 53BP1 depletion (Bouwman et al, 2010) and similarly, DNA ligase IV (lig4) deletion does not rescue Brca1 deficiency (Patel et al, 2011). Mice expressing $Brca1^{\Delta 11/\Delta 11}$ allele are embryonic lethal, show high tumorigenicity and extensive chromosomal abnormalities but this can be rescued by 53BP1 deletion (Bunting et al, 2010; Cao et al, 2009). Thus, this clearly deomnstrates that BRCA1 and 53BP1 work at the same step to drive repair commitment in opposite directions. 53BP1 accumulates in IRIF in an MDC1 and yH2AX-dependent manner and it is possible that yH2AX recruits 53BP1 to inhibit DSB resection (Reina-San-Martin et al, 2004). It associates with the dimethyl-histone H4 Lys20 (H4K20me2) chromatin modification and this inhibits DSB resection (Bothmer et al, 2010; Botuyan et al, 2006; Difilippantonio et al, 2008). It is speculated that it promotes NHEJ by promoting synapsis, which is bringing together of the two distal DSB ends, and preventing the nucleases from accessing the DSB ends. To achieve this 53BP1 requires its oligomerization domain, methyl-histone binding domain (tudor domain) and damage-induced hyperphosphorylation. But this pro-NHEJ environment is challenged by the presence of BRCA1. BRCA1 deficiency leads to genome instability in humans and mice (Moynahan et al, 1999) and it was found to act upstream of resection promoting all resection-dependent repair pathways (Stark et al, 2004).

High-resolution microscopy has shown that during S phase, 53BP1 and BRCA1 occupy mutually exclusive but associated regions in an IRIF. 53BP1 tends to be present at the periphery, whereas BRCA1 occupies the core of the foci (Chapman et al, 2012a). BRCA1 could be involved in regulating the ubiquitylation events at the break site. Although it has an intrinisic ubiquitin ligase activity, this activity seems to be dispensable for BRCA1's role in maintaining genome stability (Reid et al, 2008). BRCA1 interacts with deubiquitinating (DUB) enzymes, and by modifying the H4K20me2 pattern BRCA1 could prevent 53BP1 from enrichment at the DSB site (Lukas et al, 2011; Mallette &

Richard, 2012). DSB induced ubiquitylation is responsible for retention of pro-NHEJ factors like Ku and 53BP1 at the break site (Postow et al, 2008; Watanabe et al, 2009). Thus, it is also possible that BRCA1 signals removal of these proteins from the DSB site, tilting the balance in favor of resection. At the same time, BRCA1 might be involved in promoting HR by ubiquitylating other HR proteins or promoting protein-protein interactions.

1.3.3.2 Regulation of DSB resection during HR

During S and G2 phase of cell cycle, the primary decision maker for the fate of a DSB is resection (Ira et al, 2004; Zhang et al, 2009b). Once a DSB is formed, the Ku heterodimer can bind to the DSB much faster than HR factors (Kim et al, 2005). Thus, there is a competition between DNA end protection by Ku leading into NHEJ and DNA end resection leading into HR (Symington & Gautier, 2011). The DSB end resection is initiated by MRN and CtIP. Thus, the observation that absence of Ku relieves MRN/CtIP yeast mutants from their hypersensitivity to DNA damaging agents and the HR defect re-establishes the fact that NHEJ and HR are at the two ends of a tipping scale (Limbo et al, 2007; Tomita et al, 2003). Further insight into this matter comes from studies of meiotic recombination in yeast that is initiated by Spo11 and leads to the formation of programmed DSBs, wherein Spo11 remains attached to the 5'-end of the break. The removal of Spo11 requires endonuclease activity of Mre11 which results in nicks as far as ~300 ntds away from the 5'-DNA end followed by immediate 3'-5' exonucleolytic degradation of the nicked strand towards the break by Mre11 itself (Garcia et al, 2011). At the same time, Exo1 initiates further 5'-3' resection. This function of Mre11 has also been implicated in the release of trapped topoisomerase I and topoisomerase II complexes from DNA ends and in the removal of Ku (Hartsuiker et al, 2009; Langerak et al, 2011). Thus, this suggests that MRN has the ability to promote Exo1 mediated end resection by removing Ku from DNA ends (Buis et al, 2008). Once resection has been initiated, repair gets committed towards HDR or MMEJ because Ku binds very weakly to long ssDNA tails and trimming of the ends for ligation would require recruitment of additional proteins (Dynan & Yoo, 1998).

Although MRN complex is available throughout the cell cycle, HR takes place only during S phase when replication is going on (Karanam et al, 2012). However, NHEJ

is functional during S and G2 phase of cell cycle as well. This implies that MRN is required to remove the inhibition by Ku, but further DNA end resection is maybe regulated by other factors. Other layers of regulation come from post-translational modifications (PTMs) and protein-protein interactions. The most important PTM regulating resection is phosphorylation of DSB repair proteins by CDKs. CDKs have been shown to promote resection in yeast and vertebrates (Ira et al, 2004; Jazayeri et al, 2006), and inhibition of CDK activity in G2 prevents extensive resection, filament formation and Mec1/ATR activation (Ira et al, 2004). Levels of CDK are extremely low in G1 but they increase upon G1/S transition. Phosphorylation of CtIP by CDK in S/G2 promotes its interaction with other HR proteins like MRN and BRCA1 (Chen et al, 2008; Yu & Chen, 2004). Levels of CtIP are very low in G1 due to proteasome-mediated degradation (Germani et al, 2003), but this is prevented in S/G2 by MRN-CDK2 mediated phosphorylation of CtIP (Buis et al, 2012). MRN alone is not sufficient to initiate resection and requires the CtIP-BRCA1 complex. Phosphorylation of CtIP at Ser327 leads to formation of the MRN-CtIP-BRCA1 complex that promotes resection (Chen et al, 2008; Yun & Hiom, 2009). The phosphorylation of CtIP at Thr847 leads to its recruitment to DSBs (Huertas & Jackson, 2009).

Other proteins regulated by CDK-mediated phosphorylation are RPA and Dna2 (Ubersax et al, 2003). Phosphorylation of Dna2 promotes its nuclear localization in S phase and thus, makes it available to mediate resection at the DNA damage site (Kosugi et al, 2009). The significance of RPA2 phosphorylation is still unclear.

Another PTM involved in regulating resection is acetylation. Upon DNA damage, SIRT6, a sirtuin family member, deacetylates several constitutively acetylated residues of CtIP and this promotes HR (Kaidi et al, 2010). Other PTMS like ubiquitylation and SUMOylation have also been documented to have important roles in DSB detection, signaling and in manifestation of an appropriate DDR. The extensive crosstalk at all the steps and the intricate regulation helps to keep all the repair pathways in check to maintain genome stability.

1.3.3.3 Regulation of HR by antirecombinases

Presynaptic filament formation is the key step of HR that commits the repair of a DSB towards HR. Until the presynaptic assembly is formed, the resected DNA end can still be

channeled towards MMEJ or SSA. Regulation at the step of RAD51 assembly on the ssDNA is a very important contributor to keeping untimely HR in check. Being the most extensively studied eukaryotic model organism, budding yeast provides a lot of insight about antirecombinases, regulators of the presynaptic filament. In *Saccharomyces cerevisiae* (*S. cerevisiae)*, *srs2* mutants have a hyper-recombination phenotype and show synthetic lethality with *sgs1* mutation (Aguilera & Klein, 1988; Gangloff et al, 2000; Lee et al, 1999). Mutation of *Rad51* rescues this lethality phenotype, suggesting that the loss of viability in the *srs2 sgs1* mutant is due to unrestrained recombination leading to high levels of genome instability (Gangloff et al, 2000). Putting all the evidence together with the observation that the function of Sgs1 is to process recombination intermediates formed after dHJ formation, Srs2 was assigned the function of being an 'antirecombinase'. It negatively regulates recombination by disrupting RAD51 nucleoprotein assembly (presynaptic filaments) and thus restricting untimely HR events (Antony et al, 2009; Krejci et al, 2003; Veaute et al, 2003). Although Srs2 is a DNA helicase and belongs to the UvrD family of DNA helicases (Holthausen et al, 2010; Krejci et al, 2003), it seems to exert its antirecombinase function by functioning as a DNA motor protein (Antony et al, 2009). It interacts directly with Rad51 and stimulates its intrinsic ATPase activity, leading to dissociation of Rad51 monomers from ssDNA. Then via its translocase activity, Srs2 moves along the ssDNA to the next Rad51 monomer and again initiates the disassembly of the next Rad51monomer from the ssDNA (Antony et al, 2009).

The search for an Srs2 orthologue in mammals is still inconclusive, but there are a few possible candidates. The most potential candidate based on homology is FBH1. It belongs to the UvrD family and shows a 3'-5' DNA helicase activity (Kim et al, 2004). *Fbh1* gene disruption in chicken DT40 cells results in a mild increase in SCEs (Kohzaki et al, 2007). Also, over-expression of FBH1 in mammalian cells leads to reduced HR and a drop in RAD51 focus formation (Fugger et al, 2009), and its depletion causes an increase in RAD51 focus formation (Laulier et al, 2010). However, till date, there is no biochemical evidence to establish that FBH1 interacts directly with RAD51 and functions as an antirecombinase by disrupting RAD51-ssDNA filaments.

Another possible candidate is RECQ5. It belongs to the RecQ family of helicases and it shares many characteristic features with Srs2. Like Srs2, RECQ5 directly interacts with RAD51 and disrupts RAD51 assembly on ssDNA (Hu et al, 2007; Schwendener et al,

2010). Thus, it could regulate illegitimate HR events by preventing strand invasion and D-loop formation. Mice deficient for Recq5 have an increased predisposition to cancer and mouse embryonic stem (mES) cells show elevated levels of SCEs. Also, there is an increased persistence of RAD51 and y-H2AX foci in the mouse embryonic fibroblasts (MEFs). Another common feature between Srs2 and RECQ5 is the PCNA interaction domain (PIP motif) in their C-terminus, and both helicases have been shown to interact physically with PCNA and to have a role in replication (Kanagaraj et al, 2006; Pfander et al, 2005). Taken together, RECQ5 seems to be a potential Srs2 ortholog in mammals.

Recently, another protein, called PARI, was identified to be a key regulator of RAD51 presynaptic filament at mammalian replication forks (Moldovan, 2012 #349). PARI has an UvrD-like helicase domain, RAD51 binding site and PIP motif. Like Srs2, it interacts with SUMOylated PCNA. It is required for maintaining genomic stability in human and chicken DT40 cells, and its deficiency rescues genomic instability in Fanconi anaemia pathway deficient cells (Moldovan, 2012 #349). The only challenge in accepting it as a functional mammalian ortholoue to Srs2 is that it lacks an active helicase domain. Due to absence of Walker A or B motifs, it cannot function as a translocase like Srs2 (and RECQ5) on the ssDNA while disrupting the RAD51 assembly (Moldovan, 2012 #349). Thus, it either adopts another strategy to disrupt RAD51-ssDNA filaments or it requires the help of an accessory helicase.

BLM was also speculated to be a potential antirecombinase. It has been shown *in vitro* that BLM can disrupt inactive RAD51-ssDNA filaments (ADP bound) (Bugreev et al, 2007). But it is well established that RAD51 assembly on ssDNA requires adenosine triphosphate (ATP) for stabilization. Thus, the *in vivo* relevance of this observation remains questioned. BLM was also shown to promote strand exchange (Bugreev et al, 2009) and to be required for dHJ dissolution and hence, it regulates postsynaptic metabolism of the recombination intermediates.

Once RAD51 presynaptic filament is formed, antirecombinases can also act by disrupting the D-loop to promote SDSA. The DNA helicase Mph1, identified in yeast, serves to regulate HR post-synaptically by disrupting the Rad51-bound D-loop assembly formed after strand invasion of the homologous template (Prakash et al, 2009). Thus, it prevents crossing over and promotes non-crossover repair with short gene conversion tracts *via* SDSA.

RTEL1, identified in *Caenorhabditis elegans (C. elegans)*, can disrupt D-loops with a 3'-invasive end by unwinding synthetic strand exchange intermediates (Barber et al, 2008). Its depletion from human cells leads to a hyper-recombination phenotype and sensitivity to DNA damaging agents (Barber et al, 2008). RTEL1 deficiency in worms results in higher incidence of meiotic crossovers (Youds et al, 2010). All these observations together suggest that RTEL1 disassembles D-loops to prevent second-end capture and hence, promotes SDSA.

HELQ-1 and RFS-1 are two other proteins involved in HR regulation. Lack of these proteins in *C. elegans* does not have a marked phenotypic consequences except for a slight non-disjunction in meiosis for the *rfs-1* mutant (Yanowitz, 2008) and a small delay in resolution of RAD-51 foci for the *helq-1* mutants (Ward et al, 2010). The HELQ-1 and RFS-1 proteins have the capacity to disrupt RAD51 assembly on dsDNA and the corresponding mutants in *C.elegans* have decreased levels of meiotic COs (Ward et al, 2010). Still, more work needs to be done to understand the cellular relevance of these two helicases.

1.3.4 Physiological relevance of HR

In addition to repairing DSBs generated by endogenous or exogenous sources of damage, HR has a critical role during meiosis as it repairs the Spo11-mediated DSBs and ensures exchange of information between the maternal and paternal genetic background and thus generating diversity in the progeny (Neale & Keeney, 2006). Its importance is also reflected from the fact that Spo11 or DMC1 (Rad51 like protein in meiotic HR) deficient mice are healthy but infertile (Thompson & Schild, 2002).

Deregulation of HR often leads to an increased predisposition to cancer and premature aging due to LOH or due to copy number variation. So during HR, it is very critical to control the recombination outcome. As described previously, repair by HR leads to outcomes that are defined by parameters like gene conversion, (defined as transfer of a region of DNA sequence between two duplexes), tract length (short tract gene conversion or long tract gene conversion), and crossing over (defined as reciprocal exchange of flanking DNA sequences) (Chapman et al, 2012b). The resulting outcome is always regulated to suit the need of the cell. During meiosis, programmed DSBs are created so that the parental alleles can mix together, with at least one CO per homologous pair of

chromosome, and this results in genetic diversity (Youds et al, 2010). However, during mitosis, COs are avoided. Also, if homologous chromosome is used as a template, LOH can occur. This is not good for the cell as it can lead to dominance of recessive mutations, loss of genetic variation between the two alleles etc. (Llorente et al, 2008). If not regulated, the presynaptic filament could invade other chromosomes and this can lead to translocations in case of CO outcome (Agarwal et al, 2006). Repair of broken replication forks in regions of direct repeats like rDNA regions can drive copy number changes (Hastings et al, 2009). Thus, to prevent all the afore-stated deleterious effects of HR, the cell regulates HR by promoting short tract NCO products in mitotic cells, favoring selection of sister chromatid as template and by preventing illegitimate HR events.

The importance of HR is not just in repairing "two-ended" DSBs induced by DNA damaging agents or nucleases. HR is also very important to repair lesions encountered during replication. SSBs generated directly by DNA damaging agents like camptothecin or indirectly by the BER pathway result in stalling of replication forks. Such a SSB, when encountered by an ongoing replication fork, is converted into a one-ended DSB and requires HR for repair. If left unrepaired, it can lead to cell death.

The need for accurate and timely HR results in extensive regulation of this pathway. Its importance is also reflected from the fact that mutations in genes either directly involved in HR or regulating the pathway, lead to an increased cancer predisposition (Thompson & Schild, 2002). For example, deficiency in BRCA2 or RAD51 leads to accumulation of chromosomal aberrations over multiple cell cycles and this keeps on increasing the genomic instability and leads to establishment of cancer phenotype (Patel et al, 1998; Sonoda et al, 1998). Similarly, BLM deficient cells although proficient in HR acquire a lot of SCEs due to a shift in the balance of repair outcome. This leads to LOH and an increased susceptibility to develop cancer (Wu & Hickson, 2003). These observations hold true for many other proteins involved in HR.

The importance of HR in cells can be utilized to design effective cancer therapy. Usually cancer cells have mutations in important genes like those responsible for growth factors, apoptosis, or tumor suppressors. This situation is further worsened by the fact that most cancers accumulate mutations at a fast rate and have a high genomic instability, which is further promoted by a defective DDR system. This implies that by utilizing their defective DDR system, we can specifically target cancer cells. For example, it is seen that

cells deficient in BRCA2 are defective in repair by HR and accumulate chromosomal aberrations. Also, such cells are highly vulnerable to agents causing SSBs, as they can no more process one-ended DSBs that arise from SSBs after replication. Cells have another pathway to detect and repair SSBs and this involves PARP activity. So, if this pathway for repair of SSBs is also blocked by inhibition of PARP activity then the cell accumulates SSBs and would eventually die. At the same time, normal cells survive due to a proficient HR pathway that takes care of the SSBs. Thus, by inhibiting BER to repair a SSB lesion, the cancer cells compromised in HR can be effectively targeted (Farmer et al, 2005).

1.4 RecQ DNA helicases

DNA helicases are enzymes that convert the energy of nucleoside triphosphate hydrolysis to mechanical energy that is utilized to unwind duplex DNA in a reaction that involves enzyme translocation along ssDNA with 3'-5' or 5'-3' polarity (Patel & Donmez, 2006). The unwinding of duplex is proposed to take place in two possible ways: passive unwinding and active unwinding. In passive unwinding, the helicase is not responsible for the destabilization of the duplex rather it translocates forward and traps the ssDNA which is released temporarily due to spontaneous destabilization of the duplex. In the active mechanism, the helicase is involved in destabilizing the duplex ahead of the fork with one DNA-binding site, while at the same time holding the unwound ssDNA with a second DNA-binding site (Sharma et al, 2006).

Based on nine amino acid based sequence motifs or 'helicase signature motifs' (Q, I, Ia, Ib, II, III, IV, V and VI), helicases have been classified into six super-families (SF1-6). Motif Q is upstream of Motif I and consists of an invariant glutamine. It is involved in ATP binding. Motif I contains the Walker A box with a consensus sequence GXGKS/T. This motif forms the ATP binding site with the conserved lysine residue being involved in binding the β and γ phosphates of ATP. The Ser/Thr hydroxyl group is involved in chelating the Mg^{2+} ion required for ATP binding and consequent hydrolysis. Motif Ia is involved in binding to ssDNA. Motif II contains the Walker B box with the conserved sequence DEA/XD/H. This motif is required for interaction with Mg^{2+}, and proteins containing this motif are also referred to as DEAD-box proteins. Motif III has been shown to be involved in the coupling of ATP hydrolysis to DNA unwinding and motif VI is

required for translocation of the helicase along the DNA. Motif IV and motif I together interact directly with the nucleotide in the enzyme-ADP binary complex and motif V along with motif Ia interact directly with the oligonucleotide and the sugar-phosphate backbone (Caruthers & McKay, 2002; Tuteja & Tuteja, 2004).

The helicases belonging to the SF1 and SF2 family have a monomeric core and contain seven classical helicase motifs (I, Ia, II, III, IV, V and VI). Despite being closely related structurally, the two families differ mechanistically. Helicases belonging to SF1 family, like Srs2, translocate along ssDNA through hydrophobic interactions with the bases whereas members of the SF2 family interact with the phosphodiester backbone and this helps them in translocating on both ssDNA and dsDNA. Also, ATP hydrolysis in SF1 helicases is stimulated only by ssDNA whereas both ssDNA and dsDNA stimulate the ATPase activity of SF2 family helicases.

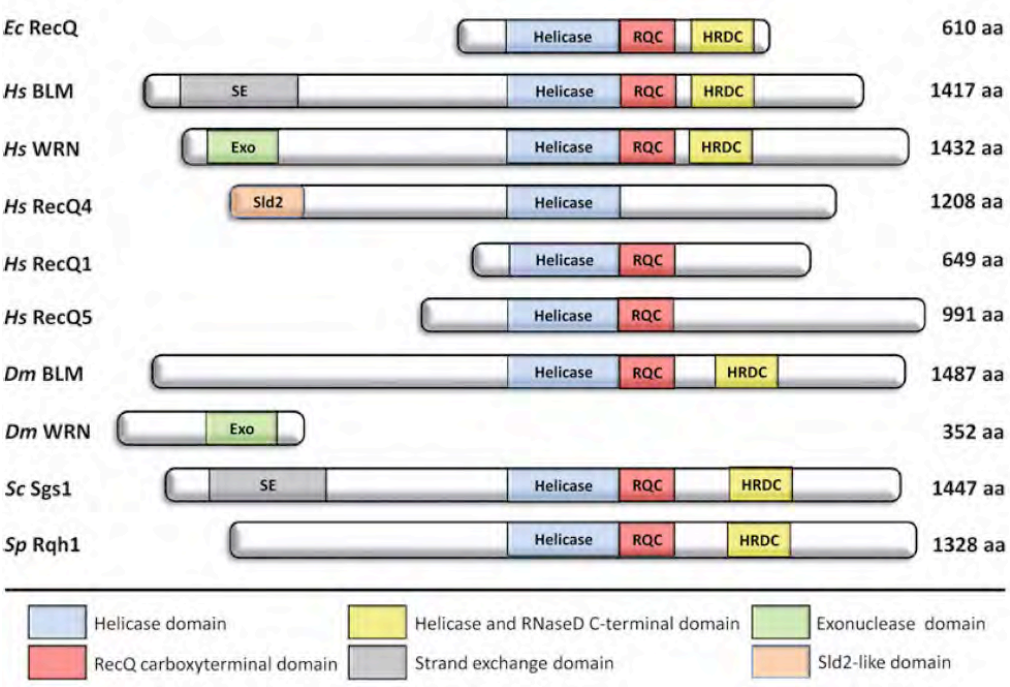


Figure 6: **Structural features of selected members of the RecQ family of DNA helicases.** Moving from unicellular prokaryotes to higher eukaryotes, the helicase domain is highly conserved but the other domains are not. RECQ1 and RECQ5 have lost the HRDC domain and RECQ4 has lost both the RQC and HRDC domain. Some RecQ helicases have taken up new domains/motifs in higher eukaryotes. The number of amino acids in each protein is indicated on the right. The abbreviations on the left stand for: *Ec, E. coli*; *Hs, H. sapiens*; *Dm, D. melanogaster*; *Sc, S. cerevisiae*. Figure Adapted from Larsen and Hickson, 2013 (Larsen & Hickson, 2013).

The RecQ DNA helicase family takes its name from the *recQ*$^{+}$ gene product in *E. coli* that was identified almost 30 years ago in a screen for mutations that conferred resistance to thymine starvation (Bachrati & Hickson, 2003; Nakayama et al, 1984). It is one of the most conserved families of DNA helicases with bacteria and lower eukaryotes like yeast having a single RecQ homolog and higher eukaryotes having multiple family members. For example, there are five RecQ helicases in *Drosophila melanogaster*, four in *C. elegans*, five in *Homo sapiens*, and seven in *Arabidopsis thaliana*. Over years, much work has been done to understand the importance of this family of DNA helicases and now they are regarded as the caretaker of the genome and help preserve genome stability. The study of this group of helicases also gained prominence due to identification of germline mutations in three of the human RecQ family genes as a cause of rare autosomal recessive disorders characterized by cancer predisposition and/or premature aging (Hickson, 2003). Mutations in *BLM* give rise to Bloom's syndrome and mutations in *WRN* give rise to Werner's syndrome (Harrigan & Bohr, 2003). Mutations in RECQ4 can result in three disorders, namely Rothmund-Thomson syndrome (RTS), RAPADILINO, and Baller-Gerold syndrome (BGS) (Harrigan & Bohr, 2003; Van Maldergem et al, 2006).

The RecQ DNA helicases belong to the SF2 family and contain all seven helicase motifs that are involved in coupling nucleoside triphosphate hydrolysis to dsDNA seperation (Bachrati & Hickson, 2003). In addition to the classical motifs, RecQ helicases may also contain two additional motifs, namely the RQC (RecQ C-terminal) and the HRDC (Helicase and RNaseD C-terminal) domains. The RQC domain is unique to RecQ helicases and is composed of a zinc-binding motif and a helix-turn-helix motif, also called the winged-helix (WH) subdomain. Four conserved cysteine residues characterize the zinc-binding motif and this motif is important for the structural stability of the protein. Mutations in these residues lead to insolubility and degradation of *E. coli* RecQ and BLM. Also, mutations in these cysteines in BLM are sufficient to cause Bloom's syndrome (Bernstein et al, 2003; Janscak et al, 2003; Liu et al, 2004). The WH subdomain forms a helix-turn-helix motif and one of these helices is important for dsDNA binding (Bernstein et al, 2003). However, this domain has no effect on the helicase activity of the protein.

The next conserved domain found exclusively in RecQ helicases, but not in all, is the HRDC domain. It is always found in the C-terminal portion of the protein and is involved in DNA binding (Bernstein et al, 2003; Janscak et al, 2003; Liu et al, 2004).

There is also evidence suggesting that this domain can confer DNA structure specificity to the helicase. For example the HRDC domain of BLM is required for the recognition and unwinding of dHJ structures, but not for unwinding of a simple partial duplex (Wu et al, 2005). The additional regions flanking the helicase, RQC, and HRDC domains are usually involved in mediating protein-protein interactions or include nuclear localization signals (NLS), post-translational modification sites etc. (Bachrati & Hickson, 2003). In some RecQ helicases, the flanking domains confer them with unique functions, for example the N-terminus of WRN harbors an exonuclease domain (Perry et al, 2006).

RecQ helicases, except for RECQ4, can unwind dsDNA in 3'-5' direction in the presence of Mg^{2+} and ATP. Some of the RecQ family members can also mediate annealing of complementary strands of DNA and some can efficiently branch migrate HJs. Many RecQ helicases can also act on special DNA structures like forked DNA, G4-quadruplex DNA, triplex DNA etc (Bachrati & Hickson, 2003). Some RecQ helicases can even displace proteins from DNA (Hu et al, 2007).

1.4.1 RecQ helicases: From *E. coli* to humans

As mentioned above, RecQ helicases were first discovered and described in *E. coli*. It was observed that mutations in *recQ*$^+$ helped survive thymine starvation (Nakayama, 2005; Nakayama et al, 1984). The RecQ protein has 610 amino acids and a classical helicase domain along with RQC and HRDC domain. In comparison to eukaryotic RecQ family members, *E. coli* RecQ has a much wider substrate specificity and higher processivity *in vitro* (Bachrati & Hickson, 2003). It does not require free DNA ends to initiate DNA unwinding and can unwind long partial duplexes as well as blunt ended duplexes (Harmon & Kowalczykowski, 2001). Amongst the many structures it can act upon, it can unwind different forked structures, HJs, G-quadruplexe DNA, and D-loops etc. *E. coli* RecQ is even more efficient in the presence of SSB, a ssDNA binding protein, possibly due to sequestration of the unwound ssDNA by SSB (Harmon & Kowalczykowski, 2001; Umezu & Nakayama, 1993). Being the sole representative of the RecQ family in *E. coli*, RecQ has been associated with various cellular functions. For example, RecQ can unwind duplex DNA resulting in ssDNA tails that can be utilized by RecA to mediate strand exchange during HR. Thus, RecQ can initiate recombination in concert with SSB and RecA (Harmon & Kowalczykowski, 1998). But at the same time, RecQ can act on D-loop

structures and thus prevent recombination. This is further substantiated by the observation that mutations in the *RecQ* gene result in a 20-300 fold increase in the frequency of recombination events (Heyer, 2004). RecQ also co-operates with RecJ and RecQ to mediate degradation of the nascent lagging strand at a stalled replication fork. This results in generation of ssDNA that is utilized by RecA to initiate strand exchange, and this also promotes activation of the SOS response pathway (Hishida et al, 2004). Furthermore, *E. coli* RecQ also interacts with topoisomerase III (top3) and stimulates its decatentation activity (Harmon et al, 1999). Thus, together they are needed for maintaining genome stability, for example at sites of converging replication forks (Suski & Marians, 2008).

In budding yeast, *S. cerevisiae*, there is only one RecQ helicase, namely Sgs1. It was isolated in a screen for suppressors of the slow growth phenotype caused by top3 mutation (Gangloff et al, 1994). Defects in the *SGS1* gene lead to sensitivity to UV, methylmethane sulfonate (MMS) and inhibitors of replication like hydroxyurea and to an elevated level of recombination events and SCEs. Sgs1 has been shown to bind and unwind various branched DNA structures (Bennett et al, 1999). It can also unwind DNA/RNA hybrids. Expression of Sgs1 is cell cycle regulated and it peaks in S phase. During S phase, Sgs1 localizes to sites of replication. Upon replication stress, Sgs1 is involved in the activation of the intra-S phase checkpoint by recruiting the effector kinase, Rad53, to the site of stalled replication fork (Frei & Gasser, 2000). Another function of Sgs1 is stabilizing stalled replication forks and preventing polα and polε dissociation from the replisome (Cobb et al, 2003). This activity of Sgs1 is independent of Rad53 and probably promotes re-establishment of replication through Mec1 and RP-A mediated polymerase association. Furthermore, Sgs1 interacts with all three topoisomerases (Top1, Top2, and Top3). A ternary complex between Sgs1-Top3-Rmi is involved in dissolution of dHJs and thus suppressing COs during HR (Cejka et al, 2012; Ira et al, 2003). Another function of Sgs1 is in the early steps of HR where along with Dna2 it promotes DSB resection (Ashton & Hickson, 2010; Cejka et al, 2010).

The orthologue of RecQ helicases in fission yeast, *Saccharomyces pombe*, is Rqh1 (RecQ type DNA helicase). A defect in the *rqh1* gene leads to slow growth rate, decreases viability and hypersensitivity to DSB causing agents such as MMS, HU, camptothecin, IR (unlike sgs1 mutants), UV etc (Doe et al, 2002; Wang et al, 2001). Both Sgs1 and Rqh1 have similar cellular roles for example, Rqh1 also associates with Top3 and Rmi1 to

dissolve dHJs and thus suppress CO products during HR. Finally, both of them also have antirecombinogenic roles because their deletion leads to a hyper-recombination phenotype in mitotic cells. To sum up, the above-described functions for Sgs1 and Rqh1 are just a fraction of their proposed cellular roles and reflect their importance in maintaining genome stability.

In humans and mice, RecQ family has expanded to include five members namely RECQ1, BLM, WRN, RECQ4, and RECQ5. The multiple family members have both unique and overlapping functions in the DNA metabolic processes and the redundancy may work to compensate in absence of one protein. Deficiency of RECQ1 is not linked with any disorder but it is important for genome stability (Singh et al, 2009). It is the smallest family member and presence of RPA stimulates its helicase activity (Cui et al, 2003). In absence of RECQ1, cells accumulate more spontaneous DSBs and have elevated levels of SCEs. They are also sensitive to DSB causing agents like IR or camptothecin. Interestingly, RECQ1 was found to interact with RAD51 and thus is involved in regulation of DSB repair by HR (Sharma & Brosh, 2007). It also shows a unique ATPase dependent 3'-5' branch migration activity which might be useful to disrupt dead-end intermediates during HR (Bugreev et al, 2008). RECQ1 is also important for replication fork stability. It can catalyze strand exchange leading to fork reversal at stalled replication forks and thus preventing them from collapsing (Popuri et al).

The second RecQ helicase family member in humans is BLM. Its deficiency causes an autosomal recessive disorder, Bloom syndrome, characterized by sensitivity to sunlight, growth retardation and predisposition to cancer (Singh et al, 2009). Cells from BS cells show exceptionally high levels of SCEs and LOH due to a loss in regulation of HR. BLM has multiple roles in HR. It is required for resection and creation of a 3'-ssDNA tail, which can then initiate HR (Nimonkar et al, 2011). It is proposed that BLM can function as an antirecombinase either by disassembling the RAD51 presynaptic filaments or by disrupting D-loops (Bugreev et al, 2007). Another important function for BLM during late HR is dissolution of the dHJ structure (Hu et al, 2001; Wu & Hickson, 2003). BLM forms a ternary complex with TOPO IIIα and BLAP75 that can efficiently act on dHJs and promote NCO product formation. Furthermore, BLM also acts at stalled replication forks. It rescues stalled replication forks by either promoting fork regression that results in a chicken-foot structure, which initiates template switching and helps bypass the lesion, or

by resolving HR-dependent replication intermediates, which appear like HJs, through branch migration (Ralf et al, 2006; Singh et al, 2009).

WRN is the next RecQ helicase family member and its deficiency causes an autosomal recessive disease characterized by premature aging, remarkably high genome instability and a predisposition to cancer. Fibroblasts from WS patients show diminished replication potential and they proceed into senescence prematurely. They also accumulate a lot of chromosomal aberrations like deletions and translocations. WRN is involved in a wide range of cellular activities. It is involved in DSB repair and its absence promotes a highly mutagenic alternative-NHEJ pathway (Sallmyr et al, 2008). WRN physically interacts with XRCC4-DNA ligase IV complex and this probably stimulates its exonuclease activity (Kusumoto et al, 2008). It also interacts with replication proteins like RPA, PCNA, and polδ. WRN colocalizes with RAD52 at sites of stalled replication forks and can stimulate the strand annealing activity of RAD52 *in vitro* (Baynton et al, 2003). So WRN can prevent collapse of a replication fork and formation of a DSB. WRN also has a role in recovery of stalled replication forks in an ATM/ATR dependent manner. It is required for ATM activation and for the downstream signaling in the event of a collapsed replication fork (Cheng et al, 2008). It is also observed that WRN can catalyze fork regression and promote HJ formation on a stalled replication fork in an ATP dependent manner (Machwe et al, 2007). Another function of WRN is in repair of ICLs and restoring normal replication forks. WRN can remove unresolved HJ intermediates by unwinding them (Bachrati & Hickson, 2003). Furthermore, WRN has a very important role in telomere maintenance and preservation. WRN interacts with telomeric proteins and its absence promotes telomeric-SCEs (T-SCEs). Further, it promotes lagging strand synthesis at telomeres by disrupting G-quadruplexes (Crabbe et al, 2004). WRN also interacts with proteins of the BER machinery and one amongst its many possible roles during BER is stimulation of the strand displacement activity of polβ (Rossi et al, 2010). These are just a brief glimpse of the many activities/roles of WRN in the cell.

Germ line defects in the *RECQ4* gene can cause three different autosomal recessive disorders, namely RTS, RAPADILINO, and BGS. RTS is characterized by a wide variety of defects like poikiloderma, growth retardation, juvenile cataracts, premature aging, and a predisposition to malignant tumors (Ralf et al, 2006; Singh et al, 2009). Cells derived from RTS patients show genomic instability and chromosomal abnormalities like

aneuploidy, trisomy, and rearrangements. The expression of RECQ4 is cell cycle regulated and it localizes both in the nucleus and cytoplasm. In the cytoplasm, it forms a complex with ubiquitin ligases, UBR1 and UBR2, which might be responsible for proteasomal degradation of RECQ4 or its interactors from the DNA replication machinery or cohesin establishment complex. Within the nucleus, RECQ4 interacts with DNA replication factors like MCM2-7, MCM10, CDC45, and GINS. It also interacts with circadian machinery protein TIMELESS and with TIPIN that is required for replication progression, stress response and establishment of cohesin (Liu, 2010).

RECQ5 is the last mammalian member of the RecQ helicase family, and it is discussed in detail in the next section.

1.4.2 RECQ5 in maintenenace of genome stability

Though RECQ5 deficiency in humans has not been linked to any heritable or acquired disease, studies in mice show that loss of RECQ5 function leads to genomic instability and predisposition to cancer (Aygun & Svejstrup, 2010; Hu et al, 2010; Hu et al, 2007). The human *RECQ5* gene was cloned in 1998, and later it was found that RECQ5 exists in three isoforms, namely RECQ5α, RECQ5β, and RECQ5γ (Sekelsky et al, 1999; Shimamoto et al, 2000). These isoforms result from alternative splicing and have predicted molecular weights of 46 kDa, 109 kDa, and 49 kDa respectively. Structurally, these isoforms share the N-terminal region spanning amino acids 1-410 that constitute the helicase domain. The shortest isoform is RECQ5α and it contains just the helicase domain. The largest isoform, RECQ5β (nowadays referred to as RECQ5) begins with the helicase domain and a putative RQC domain that is followed by a C-terminal region unique to RECQ5. It lacks the HRDC domain and the WH domain. RECQ5β has been shown to localize in the nucleus due to the presence of a nuclear localization signal (NLS) in the C-terminus (Shimamoto et al, 2000). Despite the presence of the helicase domain, RECQ5α and RECQ5γ lack any detectable helicase activity are thought to play no role in nuclear DNA metabolism due to two reasons; Firstly, RECQ5β contains a Zinc-binding motif that is important for DNA binding and ATP dependent 3'-5' helicase activity (Garcia et al, 2004), but this motif is not present in the other two isoforms. Secondly, these isoforms also lack an NLS and are seen to be present only in the cytoplasm (Kanagaraj et al, 2006; Shimamoto et al, 2000). RECQ5

expression is cell cycle independent and it is even found in G0 cells, unlike other RecQ members (Kawabe et al, 2000).

Biochemically, RECQ5 is a classical SF2 family helicase with a low processivity *in vitro,* but the presence of RPA stimulates its helicase activity. It exists as a monomer in solution and exerts its function in the same form. It was the first helicase to be shown to possess an intrinsic ssDNA-annealing activity (Garcia et al, 2004). The annealing activity resides in the non-conserved C-terminal domain of the protein and is inhibited in the presence of RPA. The physiological role of this activity is still unknown. Early biochemical studies also showed that RECQ5 could catalyze branch migration of synthetic HJs (Garcia et al, 2004).

First insight into the cellular function of RECQ5 came from the observation that RECQ5 interacts with TOPO IIIα and TOPO IIIβ (Shimamoto et al, 2000). It was thought that RECQ5 might be redundant with BLM in dHJ dissolution, but later it was found that RECQ5β-TOPO IIIα complex could not dissolve dHJs (Wu et al, 2005). Recently, it has been shown that RECQ5 also interacts with TOPO IIα and stimulates its decatenation activity (Ramamoorthy et al, 2011). Based on this finding it was proposed that RECQ5 might promote proper segregation of sister chromatids post-replication in a manner similar to yeast Sgs1 (Ramamoorthy et al, 2011; Watt et al, 1995).

The first evidence for the role of RECQ5 in DSB repair came from studies in chicken DT40 cells where it was observed that depletion of RECQ5 alone does not result in an increase in SCEs, but co-depletion with BLM results in enhanced levels of SCEs, even more than in cells with BLM depletion alone (Wang et al, 2003). The same phenotype was observed in mES cells where knock-out cells for both BLM and RECQ5 show much higher levels of SCEs compared to BLM knock-out alone (Hu et al, 2005). This epistasis analysis suggested that RECQ5 and BLM are not redundant instead they work in two different pathways to suppress COs. More evidence for the biological function of RECQ5 came from studies in mice. It was observed that *RECQ5*$^{-/-}$ knockout mice had a higher susceptibility to cancer than wild-type littermates. Cells depleted of RECQ5 showed elevated levels of chromosomal abnormalities and spontaneous DSBs in response to replication stress. Also, it was shown biochemically that RECQ5 prevents D-loop formation by the RAD51-ssDNA filament (Hu et al, 2007). It was then found that RECQ5 interacts physically with RAD51 and promotes disassembly of the ATP-bound form of

RAD51-ssDNA filament in a manned dependent on its ssDNA translocase activity (Hu et al, 2007; Schwendener et al, 2010). This put forward the model that that RECQ5 is an antirecombinase and it works by disrupting RAD51-ssDNA filaments. Thus it prevents untimely HR events and helps to maintain genomic stability in cells.

RECQ5 has also been reported to interact with the MRN complex. It was observed that RECQ5 binds to the MRE11 and NBS1 subunit and that this interaction is responsible for the recruitment of RECQ5 to sites of DSBs and stalled replication forks. It was also shown that RECQ5 inhibits the 3'-5' exonuclease activity of MRE11 (Zheng et al, 2009).

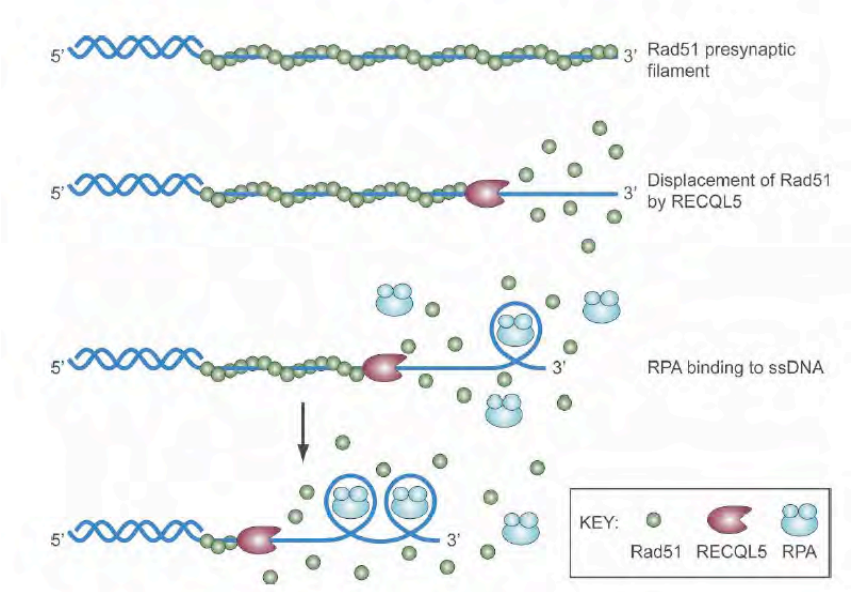


Figure 7: **Model illustrating the role of RECQ5 in HR**. RECQ5 (here RECQL5) interacts directly with RAD51 and promotes the removal of RAD51 from the ssDNA end at the break site. Once the filament has been disrupted, re-loading of RAD51 is prevented due to immediate binding of RPA to the ssDNA. Figure adapted from Hu et al, 2007 (Hu et al, 2007).

In humans, RECQ5 has been shown to interact with PCNA at sites of stalled replication forks (induced by hydroxyurea treatment). The same study showed that RECQ5 could unwind the lagging-strand arm of a synthetic fork structure, resembling a stalled replication fork, and promote strand exchange in the presence of RPA (Kanagaraj et al, 2006). This postulated a role for RECQ5 in re-activation of stalled replication forks. Another evidence for this comes from the observation that RECQ5 strongly stimulates the 5'-flap cleavage activity of the flap endonuclease 1 (FEN1). Other RECQ helicases, WRN,

BLM and RECQ4 also possess this activity and it is proposed that RECQ5 might promote restart of stalled replication forks by translocating to the fork and stimulating FEN1 to remove the Okazaki fragment, preventing DNA breakage and recombination at the stalled replication fork site (Speina et al, 2010). RECQ5 has also been reported to interact with RNA polymerase II (RNAPII) and accumulate at sites of active transcription (Aygun et al, 2009; Islam et al; Kanagaraj et al, 2010); (Izumikawa et al, 2008). So far, RECQ5 is the only RecQ helicase family member that interacts with RNAPII. It has been shown that RECQ5 interacts with the initiating and elongating form of RNAPII. It was observed that RECQ5 requires both its helicase activity and the ability to interact with RNAPII to suppress SCEs (Islam et al, 2010). Thus, RECQ5 probably is needed to regulate transcription-associated recombination events.

Although RECQ5 is a tumor suppressor and it helps in suppressing HR, its absence leads to formation of DSBs in the cell. This was an enigma for some time but recent evidence suggests that RECQ5 is also somehow involved in the suppression transcription-associated DSBs during replication (Li et al, 2011). In cells lacking RECQ5, transcription machinery is still functional and DSBs are formed, but upon inhibition of transcription, DSB formation does not occur. Further, it was observed that this effect is more pronounced in S-phase cells, suggesting that RECQ5 might acts as a regulator of genomic stability at the interface between transcription and replication. However, more work needs to be done in this direction to elucidate the mechanism by which RECQ5 prevents conflicts between the two machineries.

Collectively, all these studies suggest that RECQ5 is involved in various DNA metabolic activities encompassing DNA replication, transcription and repair.

2 AIMS

RecQ DNA helicases are considered to be guardians of the genome. In humans, there are five helicases belonging to the RecQ family and germ-line defects in three of them, namely BLM, WRN, and RECQ4, cause severe disorders associated with cancer predisposition. RECQ5 is also believed to be a tumor suppressor as its deficiency in mice leads to cancer susceptibility. RECQ5 is a 3'-5' DNA helicase that physically interacts with RAD51 and has the ability to disrupt RAD51-ssDNA filaments. However, the cellular relevance and the molecular mechanism underlying this enzymatic activity are still not understood. The aim of this PhD study was to further our understanding of the role of RECQ5 in HR. For this, we pursued the following questions.

2.1 Molecular mechanism underlying disruption of RAD51-ssDNA filaments by RECQ5

In an attempt to elucidate the mechanism of RECQ5-mediated disruption of RAD51-ssDNA filaments, we started with characterizing RECQ5-RAD51 interaction. Using mutagenesis and affinity pull-down assays, we mapped the region of RECQ5 involved in RAD51 binding and identified amino acid residues essential for this interaction. Next we investigated whether the RAD51 interaction domain of RECQ5 is important for its antirecombinase activity and its recruitment to sites of DSBs in the cell. We also mapped another region on RECQ5 that contributes towards its filament disruption activity. The results obtained in this study were partly published in Journal of Biological Chemistry. This research paper is included in the Results chapter (3.1) and the additional results are described in the section 3.2.

2.2 Role of RECQ5 in HR-mediated DSB repair

Having established that RECQ5 disrupts RAD51-ssDNA filaments through direct interaction with RAD51, we investigated the role of RECQ5 in DSB repair by HR and SSA pathways using specific GFP-based reporters integrated in human cells. We also

established biochemical assays to study the effect of RAD51 and RECQ5 on the annealing step of SSA and SDSA pathway. Finally, we investigated the effect of RECQ5 deficiency on the frequency of sister chromatid exchanges in order to assess the role for RECQ5 in suppression of mitotic crossovers. The results obtained from these experiments have been compiled into a manuscript that will be submitted to a peer-reviewed journal. The manuscript is included in the Results chapter (3.3).

3 RESULTS

3.1 Physical interaction of RECQ5 helicase with RAD51 facilitates its antirecombinase activity

Sybille Schwendener, Steven Raynard, **Shreya Paliwal**, Anita Cheng, Radhakrishnan Kanagaraj, Igor Shevelev, Jeremy M. Stark, Patrick Sung, Pavel Janscak

I contributed to this manuscript by helping in generation and purification of various deletion variants of RECQ5. Further, I tested the ability of these mutants to catalyze disruption of RAD51-ssDNA filaments. I also helped in generation of the supplementary figures and editing the manuscript.

3.2 Addition results

3.2.1 Mapping of additional domain(s) responsible for RECQ5's ability to disrupt RAD51 filaments

RATIONALE and RESULTS: As described previously in the results section 3.1, RECQ5 physically interacts with RAD51 and can disrupt the assembly of RAD51 on ssDNA. By deletion mutagenesis and pull-down assays, the RAD51 interaction domain of RECQ5 was mapped to a 70 amino acid region located within the C-terminal half of the RECQ5 polypeptide that is dispensable for the helicase and ATPase activities of the enzyme (Section 2.1, Fig. 3A). Mutation within this domain, at F666, completely abolished RECQ5-RAD51 complex formation *in vitro* as well as *in vivo* and reduced the ability of RECQ5 to disrupt filaments formed by the RAD51K133R mutant that binds DNA, but does not hydrolyze ATP. Furthermore, deletion of the C-terminal half of RECQ5 (aa 476-991) almost completely abolished its RAD51 filament disruption activity but shorter C-terminal deletions ending within or C-terminal to the RAD51 interaction domain had only moderate or no effect on this activity of RECQ5. These results suggested that RECQ5 possesses an additional functional domain between the Zn-binding motif and RAD51 interaction domains (region between 476-654) that plays an essential role in RECQ5-mediated disruption of RAD51-ssDNA filaments (Figure 1A).

Ultimately, we wanted to abolish RAD51 filament disruption activity of RECQ5 by a minimal deletion of the RECQ5 polypeptide. So, we attempted to map this additional region between the Zn-binding motif and the RAD51 interaction domain, which was required for complete RAD51-ssDNA filament disruption activity. For this, we generated and purified a series of internal deletion variants of RECQ5 including Δ461-475, Δ515-568, Δ543-607, Δ569-594 and Δ571-653, which still had an intact RAD51 interaction domain and ATPase activity (schwendener, 2009). Two more variants Δ461-674 and Δ516-674 lacking larger regions, including the RAD51 interaction domain, were also generated to investigate if we could completely abolish the filament disruption activity of RECQ5. Some of these variants, along with RECQ5 and 1-475 variant, were tested for their ability to disrupt RAD51K133R-ssDNA filaments using Topoisomerase I-linked

DNA supercoiling assay (Figure 1B). The full-length RECQ5 and the variants Δ461-475 and Δ571-653 showed comparable supercoiled dsDNA product formation, but the variants Δ519-568, Δ543-607, and Δ569-594 demonstrated compromised filament disruption activity (Figure 1D). Taken together, these results suggest that the region between aa 476-570 harbors another domain that contributes towards RECQ5's ability to disrupt RAD51-ssDNA filaments.

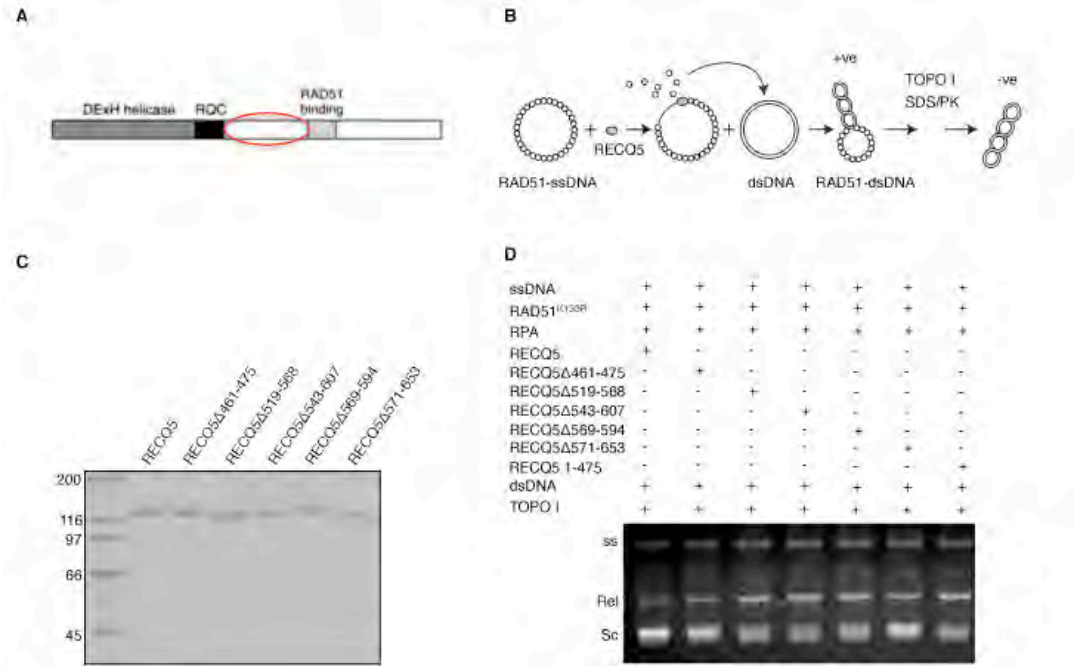


Figure X: **Analysis of RAD51K133R-filament disruption activity of RECQ5 variants.** (A) Scheme of RECQ5. The red oval depicts the region of RECQ5 being analyzed for its contribution towards RECQ5's filament disruption activity. (B) The scheme of the Topoisomerase I-linked DNA supercoiling assay. Adapted from (Colavito et al, 2010). (C) Commassie stained SDS-PAGE to verify the purity and concentration of the variants used in the study. The molecular weights of the marker proteins are indicated on the left. (D) The RAD51K133R-ssDNA filament was pre-formed, using 1.5 μM of RAD51K133R and 6 μM of ssDNA (in ntds), by a 6 min incubation at 37 °C. This was followed by addition of 40 nM RPA and 160 nM of either RECQ5 or one of the variants. After incubation for 8 min, 7 μM dsDNA (in bp) and 3 U of wheat germ TOPO I were added and the reaction mixture was incubated at 37 °C for another 8 mins. The reactions were stopped using SDS and proteinase K and resolved in a 1% agarose gel followed by visulaization of products using Ethidium Bromide stain. RQC, RecQ C-terminal domain; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TOPO I, Topoisomerase I; SDS, sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURE: Construction of the following variants 1-475, Δ461-475, Δ515-568, Δ543-607, Δ569-594 and Δ571-653 are described in the thesis of S. Schwendener (schwendener, 2009). The recombinant proteins were run on an 8% SDS-

PAGE to verify their purity and ensure their concentration. The gel was subjected to Commassie blue staining. The ATPase assay and the Topoisomerase I-linked DNA supercoiling assay have been described under materials and methods in section 3.1.

The variant Δ461-674 was generated by ligation of the larger fragment resulting from Acc651/PvuI digestion of pPG10Δ461-475 with the smaller fragment resulting from Acc651/PvuI digestion of pPG10Δ652-674. The Δ516-674 variant was constructed by cleaving pPG10Δ652-674 with Acc651, followed by Klenow treatment, restriction digestion with Fsp1, and self-ligation. Both of these variants were purified as described previously (schwendener, 2009).

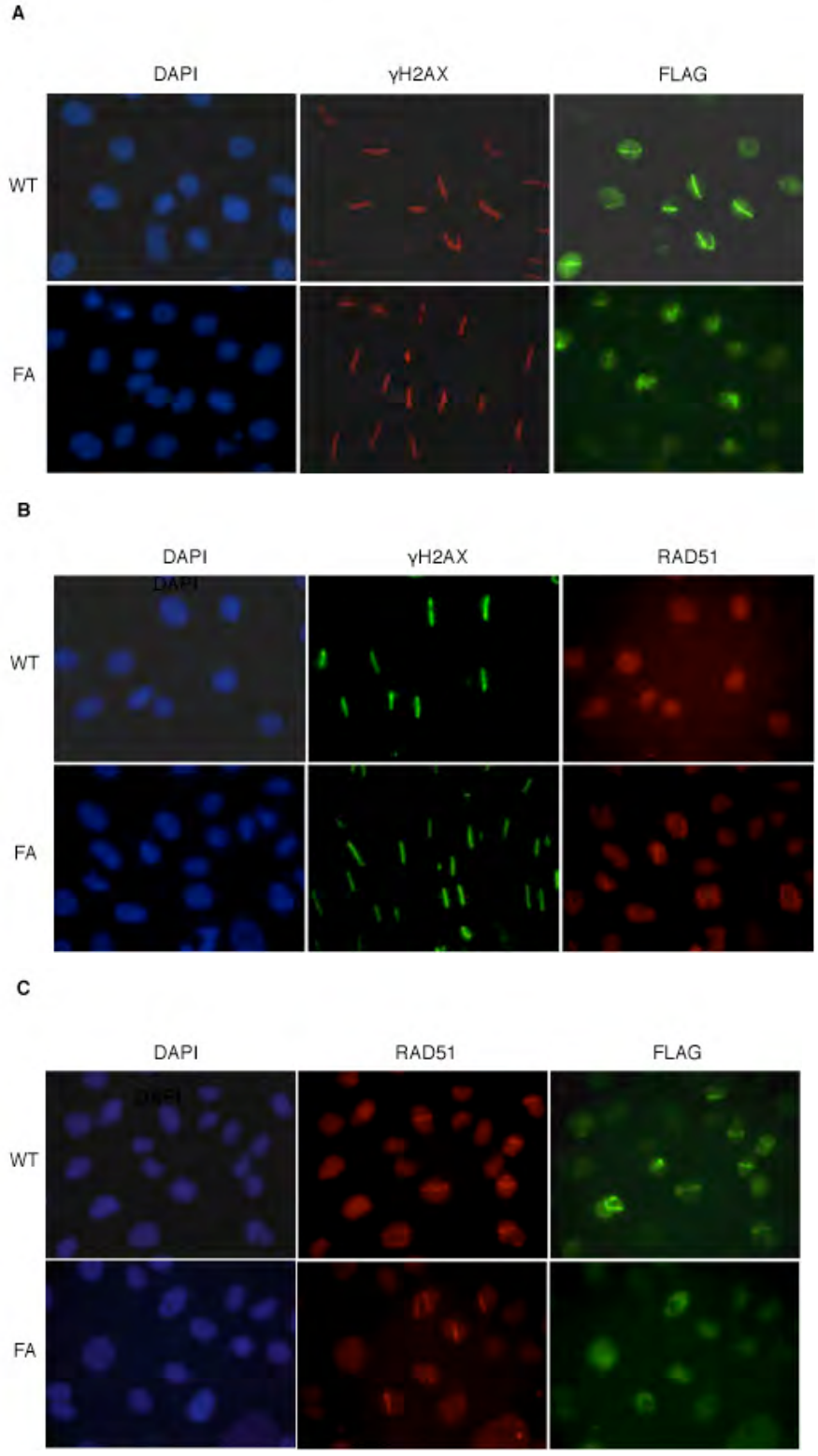
3.2.2 Analysis of recruitment of RECQ5 WT and RECQ5 F666A mutant to laser lines

RATIONALE and RESULTS: Previously we have established that the F666 residue of RECQ5 is essential for RAD51 binding. Next we wanted to analyze the *in vivo* relevance of this interaction. It has been observed that RECQ5 is recruited very early to laser induced DSBs (Popuri et al, 2012b; Zheng et al, 2009). Hence, we investigated the role of RECQ5-RAD51 interaction in the recruitment of RECQ5 to sites of laser-induced damage (referred to as laser stripes). In our institute, we have a special U2OS TREx cell line with a chromosomally transformed cassette derived from the pAIO (**pA**ll-**I**n-**O**ne) vector. The cassette is under tetracycline regulation and upon induction with tetracycline (or doxycycline, Dox), it expresses an shRNA against a gene-of-interest and an shRNA resistant form of that gene (Kehl, 2009). Utilizing the same set-up, we have cell lines for inducible expression of recombinant wild type RECQ5 or its mutant, RECQ5 F666A (FA), with a C-terminal 3x-FLAG tag (Hühn, 2010). There are two advantages of using these cell lines; Firstly, they are inducible and depending upon the amount of Dox used and the time span of induction, the levels of recombinant RECQ5 protein in the cell can be regulated. Thus we can avoid the dominant negative effect, if any, from the recombinant RECQ5 protein. Secondly, simultaneous expression of the shRNA, that removes the endogenous RECQ5, promotes the preferential recruitment of recombinant protein to sites of RECQ5 function.

Upon laser micro-irradiation, we could confirm the presence of DSBs by detection of a stripe-like signal from γH2AX antibody (Figure 2A). Cells that were not subjected to

irradiation were checked for homogeneity in expression of FLAG tagged recombinant protein, both WT and FA. Almost 90% of the cells expressed the recombinant proteins but the level of expression varied amongst the cells. Upon co-immunostaining with γH2AX and FLAG antibodies, we observed that in WT cells, 63% of the γH2AX lines showed accumulation of FLAG-RECQ5 WT, whereas in FA cells only 28% of the γH2AX lines showed accumulation of FLAG-RECQ5 FA (Figure 2 A & 2D). This implied that recruitment of RECQ5 to DSBs is dependent on its interaction with RAD51.

To gain further insight into the differential recruitment pattern of RECQ5 WT and RECQ5 FA, we co-immunostained the irradiated cover slips with RAD51 and FLAG antibodies. Another set of cover slips were co-immunostained with γH2AX and RAD51 antibodies to confirm the recruitment of RAD51 to γ H2AX lines (Figure 2B & 2E). Strikingly and much to our expectation, only 8% of the RAD51 lines in FA cells showed recruitment of FLAG-RECQ5 FA, where as in WT cells, 42% RAD51 lines showed recruitment of WT RECQ5 (Figure 2C & 2F). Further analysis of RAD51 and FLAG stained cover slips revealed that in both WT and FA cells, FLAG tagged both WT and FA RECQ5 accumulate at laser stripes lacking RAD51 which most likely represent G1 cells. Thus our data strongly suggests that recruitment of RECQ5 to DSBs in S-phase cells is dependent on RAD51. Mutating the critical residue, F666, required for RAD51 interaction (Schwendener et al, 2010) results in a drastic reduction in its recruitment to DSBs sites during S phase. This also provides explanation for the observation that in absence of RECQ5, RAD51 foci persist longer (Hu et al, 2007). Thus, recruitment of RECQ5 to S-phase DSBs, which are undergoing repair by HR, is RAD51 interaction dependent. The expression of recombinant RECQ5 in both cell lines was confirmed by western blotting and probing with FLAG antibody (Figure 2G).


A
DAPI
γH2AX
FLAG
WT
FA
B
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γH2AX
RAD51
WT
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C
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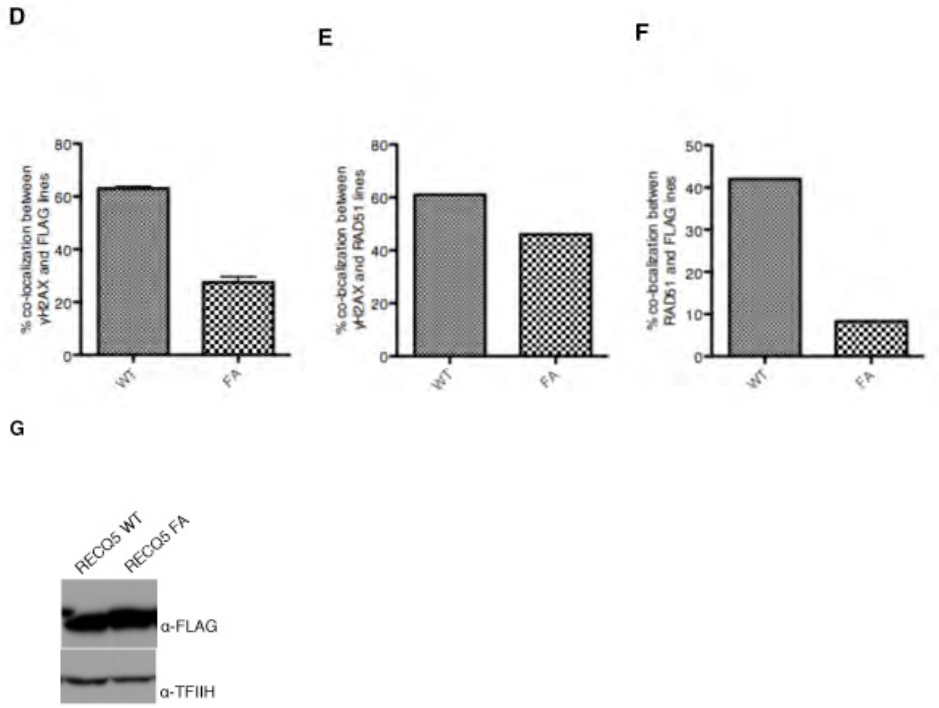

Figure 2: **Recruitment analysis of FLAG tagged WT RECQ5 and FA RECQ5 to DSBs generated by laser irradiation.** (A) Co-localization between γH2AX and FLAG at laser stripes. U2OS TREx cell lines expressing WT or FA RECQ5 were micro-irradiated to generate DNA damage. After 15 minutes, the coverslips were pre-extracted, fixed and co-immunostained with γH2AX and FLAG antibodies. Images were taken using a Leica DM6000 microscope under 63X magnification for DAPI, γH2AX and FLAG signal. (B) Co-localization between γH2AX and RAD51 at laser stripes. Same procedure was followed as described before but the coverslips were co-immunostained with γH2AX and RAD51 antibodies (C) Co-localization between RAD51 and FLAG at laser stripes. Same procedure was followed as described for (A) but the coverslips were co-immunostained with RAD51 and FLAG antibodies. (D), (E), and (F) are graphical representation of quantitative analysis of images shown in (A), (B), and (C) respectively. For each analysis, at least 30 individual cells with laser lines were counted. (G) Western-blot analysis for verifying expression of the FLAG-tagged RECQ5. Immuno-blots were probed against FLAG antibody.

EXPERIMENTAL PROCEDURE: U2OS TREx (Invitrogen) cell lines stably express the tet-repressor so the derived cell lines were propagated in DMEM supplemented with 10% Tet-system approved FBS (Sigma-Aldrich), 50 μg/ml Hygromycin B (PAA Laboratories GmbH), 100 U/ml Penicillin and 100 μg/ml streptomycin (Gibco). They were maintained in a humidified incubator containing 5% CO_2 at 37 °C. The cell lines utilized for these experiments were RECQ5 WT and RECQ5 FA and were constructed by Daniela Hühn (Hühn, 2010).

In order to generate DSBs in defined nuclear compartments, laser micro-irradiation was performed using a MMI CELLCUT system containing a 355 nm UVA

laser (Molecular Machines & Industries). Laser velocity was set at 75%, laser focus at 47.7% and laser intensity was at 55.6%. Prior to laser micro-irradiation, cells were grown on a 12 mm glass microscope coverslips (OmniLab) in appropriate culture medium containing 10 μM Bromodeoxyuridine (BrdU, Sigma) and 0.1 ng/ml Dox (Clontech) for 24 h. It has been established that 0.1 ng/ml Dox induction leads to expression of recombinant RECQ5 (WT or FA) to levels comparable to those of endogenous protein. Prior to irradiation, cells were transferred to micro-irradiation chambers (LAB-TEK) and kept in 1 ml of culture medium during micro-irradiation. A small area of the cover slip was marked for irradiation under the above-mentioned conditions. After irradiation, cells were incubated at 37 ˚C for 15 min, pre-extracted, fixed and immunostained as described below.

Post micro-irradiation, cells on all cover slips were pre-extracted for 5 min on ice in 25 mM HEPES (pH 7.4) containing 0.5% Triton X-100, 50 mM NaCl, 1 mM EDTA, 3 mM MgCl2 and 0.3 M sucrose. After washing once with phosphate buffered saline (PBS), they were fixed with 4% (v/v) Formaldehyde in PBS for 12 min followed by washing in PBS. After blocking in PBS containing 3% FCS for 30 min at RT, cover slips were incubated for 2 h at RT with appropriate primary antibodies diluted in blocking solution. After washing with PBS, they were incubated for 30 min at RT with secondary antibodies diluted in blocking solution. After washing with PBS, coverslips were mounted on Vectashield (Vector Laboratories) and images were captured at 63X or 100X magnification on Leica DM6000 fluorescence microscope.

Primary antibodies used for immuno-fluorescence staining are as follows: mouse monoclonal γ -H2AX (Millipore); rabbit monoclonal γH2AX (Cell signaling); mouse monoclonal FLAG (Sigma Aldrich); rabbit polyclonal RAD51 (lab-made). The Secondary antibodies used are Alexa Fluor 569 conjugated goat anti-rabbit IgG (Invitrogen), Alexa Fluor 488 conjugated goat anti-mouse IgG (Invitrogen).

3.3 Human RECQ5 helicase promotes repair of DNA double-strand breaks by synthesis-dependent strand annealing

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Manuscript under preparation

I contributed to this manuscript by performing all experiments (except for SCE analysis) and by analyzing the data. I improved the protocol for measuring repair of DSBs by siRNA-mediated depletion of proteins in the DR-GFP and SA-GFP reporter assays. I also established a biochemical assay to analyze the effect of different proteins on the annealing step of the SSA and SDSA pathways *in vitro*. I was also involved in purification of various proteins such as RAD52, RECQ5 and RAD51K133R used in the study. Finally, I was involved in compilation and editing of this manuscript.

Human RECQ5 helicase promotes repair of DNA double-strand breaks by synthesis-dependent strand annealing

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Running title: RECQ5 promotes SDSA

Abstract

Most mitotic homologous recombination (HR) events proceed via a synthesis-dependent strand-annealing (SDSA) mechanism to avoid crossing-over, which may give rise to chromosomal rearrangements and loss of heterozygosity. However, the molecular mechanisms controlling HR sub-pathway choice are poorly understood. Here, we provide evidence that human RECQ5, a DNA helicase that can disrupt RAD51 nucleoprotein filaments, promotes HR with non-crossover outcome by acting in a pathway, which is distinct from dissolution of double Holliday junctions. We also show that RECQ5 counteracts the inhibitory effect of RAD51 on RAD52-mediated annealing of complementary DNA strands *in vitro* and *in vivo*. Finally, we demonstrate that lack of RECQ5 causes an elevation of sister chromatid exchanges upon inactivation of the dissolution pathway or upon induction of a high load of DNA damage in the cell. Collectively, our findings suggest that RECQ5 is essential for the post-synaptic phase of SDSA to prevent formation of an aberrant RAD51 filament on the extended invading strand, thus limiting its channeling into potentially hazardous crossover pathway.

Introduction

DNA double-strand break (DSB) is the most dangerous DNA lesion in the cell since its inaccurate repair can lead to chromosomal rearrangements, a hallmark of tumorigenesis and tumor progression. In eukaryotic cells, two mechanistically distinct pathways are known to efficiently repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). In NHEJ pathway, which is operational throughout the cell cycle, DNA ends are directly ligated in an error-prone manner, frequently resulting in small deletions or insertions at the break sites, or in genomic rearrangements (Lieber, 2010). In contrast to NHEJ, HR is restricted to the S-phase and requires the presence of an intact homologous sequence to be utilized as a template for DSB repair (Karanam et al, 2012; San Filippo et al, 2008; Sung & Klein, 2006). It usually leads to restoration of the original DNA sequence if the undamaged sister chromatid serves as a homology donor. HR is initiated by nuclease-mediated resection of the DNA ends to generate 3'-single-stranded (ss) DNA tails that are coated by the ssDNA-binding protein

RPA (Symington & Gautier, 2011). In the next step, the RAD51 recombinase replaces RPA on these ssDNA tails with the help of mediators such as BRCA2 to form a nucleoprotein filament that catalyzes the invasion of the donor chromatid, giving rise to a three-stranded structure called the displacement (D)-loop. After DNA synthesis, primed by the invading strand, repair can proceed *via* two main sub-pathways referred to as the canonical DSB repair (DSBR) and synthesis-dependent strand annealing (SDSA) (San Filippo et al, 2008; Sung & Klein, 2006). In DSBR pathway, the second DNA end is captured by the D-loop to form an intermediate with two Holliday junctions, referred to as double Holliday junction (dHJ). This joint DNA molecule can be either resolved by specialized endonucleases into crossover (CO) or non-crossover (NCO) products or dissolved by the BLM-TOPOIIIα-RMI1/2 (BTR) complex, giving rise exclusively to NCO products. In the SDSA pathway, the extended D-loop is disrupted by a DNA helicase, and the newly synthesized DNA is annealed to the ssDNA tail of the other break end, which is followed by gap-filling DNA synthesis and ligation. SDSA yields exclusively NCO products (San Filippo et al, 2008; Sung & Klein, 2006).

The HR sub-pathways are under strict regulation to select the most appropriate outcome in a given state of the cell (Chapman et al, 2012b; Heyer et al, 2010). Although formation of COs is favored during meiosis to ensure genetic diversity and accurate chromosome segregation, it is suppressed in mitotic cells to prevent loss of heterozygosity and chromosomal translocations (Matos et al, 2011; Youds & Boulton, 2011). Recent studies in yeast and mammalian cells suggest that HJ resolvases are active only during M phase, biasing the outcome of recombination towards NCO products while also ensuring the elimination of any persistent joint DNA molecules (Matos et al, 2011). Most NCOs arising during HR-mediated DSB repair are produced by SDSA rather than by the canonical DSBR pathway, with dHJ dissolution accounting only for 8% of NCO events (Mitchel et al, 2010). Moreover, the resolution of HJs is highly constrained to generate CO products (Mitchel et al, 2010). Thus it appears that SDSA is the preferred pathway of HR-mediated DSB repair in mitotic cells.

In budding yeast, the Mph1 DNA helicase suppresses COs during ectopic gene conversion by acting in a pathway distinct from dHJ dissolution (Prakash et al, 2009). Mph1 influences outcome rather than the efficiency of recombinational repair events, suggesting that it acts by shunting a DNA repair intermediate into the SDSA pathway (Prakash et al, 2009). In support of this notion, biochemical evidence indicates that Mph1 is capable of disrupting Rad51-made D-loops (Prakash et al, 2009). Another suppressor of COs in yeast proposed to act via promotion of SDSA is Srs2, an UvrD-type DNA helicase that has the capacity to displace Rad51 from ssDNA. However, the mechanism of CO suppression by Srs2 appears to differ from that of Mph1. Cells lacking Srs2 display a failure to complete ectopic gene conversion with NCO outcome, which reduces the overall repair efficiency and therefore increases the proportion of crossover products among completed recombination events (Ira et al, 2003). Although Srs2 can unwind synthetic D-loop structures and DNA duplexes covered by Rad51, it fails to unwind Rad51-made D-loops (Dupaigne et al, 2008; Prakash et al, 2009). Instead, the antirecombinase activity of Srs2 *in vivo* is dependent on its ability to bind RAD51, suggesting that Srs2 might promote SDSA by regulating Rad51 filament stability (Colavito et al, 2009).

The closest sequence homologue of Srs2 in mammals and other vertebrates is FBH1, which is found in fission yeast but not in budding yeast. Several lines of *in vivo* evidence suggest that this UvrD-type helicase regulates HR at the stage of RAD51 filament assembly, but its role in SDSA is yet to be assessed (Fugger et al, 2009). Another potential orthologue of Srs2 in mammals is RECQ5, which belongs to RecQ family of DNA helicases. Biochemical studies have shown that RECQ5 binds directly to RAD51 and possesses the ability to disrupt the ATP-bound form of RAD51-ssDNA filament in a manner dependent on its ssDNA-translocase activity (Hu et al, 2007; Schwendener et al, 2010). In accordance with this finding, phenotypic analyses of mouse knockout cells have revealed that RECQ5 functions to regulate HR (Hu et al, 2005; Hu et al, 2007). Here we provide several lines of evidence suggesting that RECQ5 promotes SDSA by disrupting aberrant RAD51-ssDNA filaments formed during the post-synaptic stage of this HR sub-pathway, decreasing the risk of potentially hazardous COs.

Results and Discussion

To gain deeper insight into the biochemical mechanism underlying SDSA in mammalian

cells, we investigated the role of the two potential human orthologs of Srs2, FBH1 and RECQ5, in the formation of NCO products during HR-mediated repair of endonuclease-induced DSBs. In this analysis, we also included BLM since it was shown earlier to disrupt RAD51 nucleoprotein filament (Bugreev et al, 2007). To selectively detect NCO events, we used the DR-GFP reporter system established previously in HEK293 cells (Bennardo et al, 2008; Stark et al, 2004). This reporter consists of a direct repeat of two mutated GFP alleles: a full length GFP interrupted by a recognition site for the I-SceI endonuclease and an internal GFP fragment that serves as a donor for HR-mediated repair of the DSB created by I-SceI in the upstream GFP allele. HR-mediated repair of this DSB via a NCO event gives rise to a functional GFP allele, while repair by crossing over yields a C-terminally truncated GFP allele that does not encode for a fluorescent protein (Figure 1A). Using RNAi technology, we depleted proteins of interest in the DR-GFP reporter cell line (Figure 1B and Figure S1). Two days after siRNA transfection, cells were re-seeded in a 12-well plate and transfected with an I-SceI expression vector. The percentage of GFP-positive cells was measured by flow cytometry two days after plasmid transfection. As expected, formation of a functional GFP allele was impaired in cells depleted for the RAD51 recombinase (Figure 1B and C). Among the tested DNA helicases, only depletion of RECQ5 led to a significant reduction of repair efficiency compared to control cells without affecting cell cycle distribution (Figure 1B and C, Figure S2). Surprisingly, depletion of BLM dramatically increased repair efficiency relative to control, indicating that dHJ dissolution has no role in the formation of NCO repair products in the DR-GFP system (Figure 1B and C). Instead, BLM probably disrupts joint DNA molecules formed during the repair process. To substantiate our observations, we tested the effect of RECQ5 depletion on I-SceI-induced gene conversion in the DR-GFP reporter system established in U2OS cells (Gunn et al, 2011). Again, we found that RECQ5-deficient cells showed a reduced frequency of NCO repair events compared to control cells (Figure 1D and E). We also observed that RECQ5 depletion dramatically reduced NCO repair efficiency in HEK293/DR-GFP cells lacking BLM (Figure 1B and C). Thus our results suggest that RECQ5 might promote repair of DSBs by the SDSA pathway of HR, whereas BLM acts as a SDSA suppressor.

In budding yeast, SDSA requires Rad52 that mediates DNA strand annealing in a reaction stimulated by RPA (Paques & Haber, 1999). Similarly, we found that depletion of

human RAD52 resulted in a significant decrease in NCO repair efficiency as measured by DR-GFP reporter assay, suggesting that RAD52 is required for SDSA in human cells as well (Figure 1B and C). In order to prove that RECQ5 promotes DSB repair by SDSA, we tested the effect of co-depletion of RECQ5 and RAD52 on repair efficiency in the DR-GFP system. This analysis indicated the negative effects of depletion of these proteins on the formation of NCO products were epistatic, providing another line of evidence for the involvement of RECQ5 in SDSA (Figure 1B and C).

It is possible that RECQ5 promotes SDSA by catalyzing disruption of aberrant RAD51 filaments that might form on the newly synthesized DNA strand following unwinding of the extended D-loop. Such RAD51 filaments would inhibit the ssDNA-annealing step of SDSA and initiate reformation of the D-loop, shifting the balance between the HR sub-pathways in favor of DSBR. To explore this possibility, we utilized a GFP-based reporter for DSB repair by single-strand annealing (SSA), which mechanistically resembles the post-synaptic phase of SDSA (Bennardo et al, 2008; Stark et al, 2004). The reporter cassette, termed SA-GFP, contains two truncated GFP alleles that share a 266-bp region of homology (Figure 2A). This direct sequence repeat is separated by a region of about 2.7 kb with a I-SceI-recognition site located at the 5'-end on the distal GFP allele. Repair of the DSB created by I-SceI results in the formation of a functional GFP allele only if SSA-mediated recombination takes place between the repeated GFP sequences. We found that depletion of RAD51 resulted in a 2-fold increase in repair efficiency compared to control, indicating that RAD51 inhibits the SSA pathway of DSB repair in human cells (Figure 2B and C). On the contrary, depletion of RAD52 protein resulted in a dramatic decrease in the frequency of SSA repair events, which is consistent with the proposed role for RAD52 in promoting ssDNA annealing during SSA (Figure 2B and C) (Stark et al, 2004). Depletion of RECQ5 caused a significant reduction in SSA repair efficiency, but only in the presence of RAD51. Cells depleted for RECQ5 and RAD51 displayed a SSA repair capacity similar to that showed by cells depleted for RAD51 alone. (Figure 2B and C, and Figure S3). Thus these data strongly suggest that RECQ5 counteracts the inhibitory effect of RAD51 on DSB repair by SSA, most likely by catalyzing disruption of RAD51 filaments formed on ssDNA generated by DNA end resection.

To test this hypothesis, we investigated the effect of RAD51 on RAD52-mediated

annealing of two complementary oligonucleotides either in absence or presence of RECQ5. In these biochemical experiments, we used an ATP hydrolysis-deficient mutant of RAD51, RAD51K133R, which can form a stable nucleoprotein filament in the presence of ATP, mimicking the *in vivo* ATP-bound form of the filament that is capable of catalyzing DNA strand exchange (Hu et al, 2007). Prior to annealing reactions, a 30-mer oligonucleotide was pre-incubated with RPA to form an ssDNA-RPA complex, while the other oligonucleotide (59-mer, radioactively labeled at its 5'-end) was pre-incubated either with RAD51K133R to form a nucleoprotein filament or with the reaction buffer alone. We found that addition of RPA-coated 30-mer oligonucleotide to free 59-mer oligonucleotides in presence of RAD52 resulted in rapid formation of partial DNA duplex structure with a 3'-tail. (Figure 3A, *first panel from the right*, and Figure S4). However, this RAD52-mediated ssDNA annealing was impaired if the 59-mer oligonucleotide was pre-coated with RAD51K133R prior to its addition to the annealing reaction, demonstrating that formation of RAD51-ssDNA filaments inhibits ssDNA annealing by RAD52. Remarkably, the inhibitory effect of RAD51K133R on RAD52-mediated ssDNA annealing was almost completely lost upon addition of RECQ5 to the reaction (Figure 3A and B). On the contrary, a helicase-deficient mutant of RECQ5, RECQ5K58R, did not alleviate this inhibitory effect, suggesting that RECQ5 stimulated the ssDNA-annealing reaction by disrupting RAD51K133R-ssDNA filaments (Figure 3A and B).

To substantiate the above findings, ssDNA annealing reactions were also carried out in the presence of a homologous DNA duplex (59-mer), conditions resembling the post-synaptic stage of SDSA. We found that this DNA duplex had no effect on RAD52-mediated annealing of the two complementary oligonucleotides. However, if the 59mer oligonucleotide was pre-coated with RAD51K133R, we again observed a strong inhibition of RAD52-mediated ssDNA annealing with concomitant appearance of radioactively labeled 59-mer oligoduplex, an indicative of strand exchange reaction (Figure 3D). Upon addition of RECQ5, RAD51K133R-dependent strand exchange was inhibited and RAD52-mediated ssDNA annealing was restored to a level detected in absence of RAD51K133R. Again this effect was not seen with the K58R mutant of RECQ5. These data provide evidence that RAD51 can promote re-formation of D-loop during the post-synaptic stage of SDSA and that RECQ5 can counteract this reverse reaction by removing RAD51 from the invading strand.

Disruption of the RECQ5 gene in mouse ES cells leads to an increase in the frequency of spontaneous sister chromatid exchanges (SCEs), indicating that RECQ5 acts a CO suppressor (Hu et al, 2007). However, in chicken DT40 cells, disruption of the RECQ5 gene increased SCE frequency only in $BLM^{-/-}$ background, suggesting that RECQ5 serves as backup for BLM (Wang et al, 2003). To assess the role of human RECQ5 in suppression of mitotic COs, we investigated the effect of siRNA-mediated depletion of RECQ5 and BLM on SCE frequency in U2OS cells prior to and after induction of DSBs by camptothecin (CPT). We observed that RECQ5 depletion in untreated cells had no significant effect on the SCE frequency, whereas depletion of BLM increased the SCE frequency by almost 3-fold compared to control cells (Figure 4A and C). Cells depleted for RECQ5 and BLM showed a much higher SCE frequency than cells depleted for BLM alone (Figure 4A and C), which is consistent with the studies in chicken and mouse cells (Hu et al, 2005; Wang et al, 2003). Importantly, in the presence of CPT, a marked elevation of SCE frequency was observed not only upon BLM depletion, but also upon depletion of RECQ5, suggesting that RECQ5 has a role in CO suppression even in the presence of BLM if the load of DNA damage exceeds a certain threshold (Figure 4B). Again cells depleted for both RECQ5 and BLM exhibited a much higher frequency of CPT-induced SCEs than cells depleted for either of these proteins (Figure 4B). Thus, these data indicate that RECQ5 and BLM act in two different pathways to suppress CO formation during HR, and support our hypothesis that RECQ5 promotes SDSA. It is conceivable that at low levels of DSBs in the cell, all dHJs formed as a consequence of SDSA failure (e.g. due to RECQ5 deficiency) are dissolved by the BLM-TOPOIIIα-RMI1/2 (BTR) complex to yield NCO products. However, at high load of DSBs, the amount of dHJs formed in an SDSA-deficient cell is likely to exceed the repair capacity of the BTR complex, favoring resolution to CO products. This assumption is consistent with our finding that RECQ5-deficient cells exhibited a marked increase in SCE frequency only upon exposure to CPT (Figure 4).

Based on our data, we propose a model wherein RECQ5 acts as a RAD51 filament disruptase during the post-synaptic stage of SDSA to prevent re-formation of the D-loop, which would favor the classical DSBR pathway, increasing the risk of COs (Figure 4D). Futile cycles of D-loop disruption and reformation could also lead to cell death due to persistence of recombination intermediates. The same model is applicable for the

antirecombinase activity of Srs2 in budding yeast. Consistent with this proposal, it has been shown that over-expression of Rad51 in Δ*srs2* mutant cells nearly eliminates the NCO pathway without affecting the formation of COs, providing evidence that Rad51 inhibits the post-synaptic stage of SDSA (Ira et al, 2003). In addition, like RECQ5, Srs2 counteracts the inhibitory effect of Rad51 on DSB repair by SSA (Carter et al, 2009; Ira et al, 2003; Sugawara et al, 2000; Vaze et al, 2002). Moreover, it has been observed that over-expression of Srs2 suppresses the high level of crossovers in Δ*sgs1* cells, suggesting that Srs2 can shift the balance between DSBR and SDSA pathways in favor of the latter. It remains to be determined how is the antirecombinase activity of RECQ5 and Srs2 are regulated to prevent disassembly of the legitimate RAD51 filaments formed after DNA end resection. One possibility that deserves further investigation is that the action of these helicases on RAD51 filaments is counteracted by RAD51 mediators such as BRCA2, which facilitates filament assembly by stabilizing RAD51 binding to ssDNA. In support of this notion it has been demonstrated that the inhibitory effect of Srs2 on Rad51 focus formation in budding yeast is antagonized by Rad52, which promotes Rad51 filament assembly by a mechanism similar to that of BRCA2 (Burgess et al, 2009). Moreover, yeast Rad52 has been shown to inhibit Srs2-catalyzed Rad51-ssDNA filament disruption in vitro (Burgess et al, 2009).

Materials and Methods

Antibodies and purified proteins

Primary antibodies used for immunoblotting are as follows: rabbit polyclonal RECQ5 and RAD51 (made in the laboratory); rabbit polyclonal BLM (Abcam), rabbit polyclonal TFIIH, RAD52 and mouse monoclonal β-tubulin (Santa Cruz Biotechnology). The secondary antibodies used are sheep anti-mouse IgG-horseradish peroxidase (Sigma Aldrich) and donkey anti-rabbit IgG-horseradish peroxidase conjugates (GE Healthcare).

RAD52 was over-produced in *E. coli* BL21 (DE3) pLysS as a fusion with a hexa-histidine tag (His_6), and purified to homogeneity as described previously with minor modifications (Benson et al, 1998). Briefly, cells were collected, resuspended in 40 ml of T buffer (0.02 M Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 0.02% Triton X-100) containing 5 mM imidazole and lysed by sonication.

After clearing the lysate, it was loaded on a Ni^{2+}-charged HiTrap Chelating HP column (5 ml; GE Healthcare) equilibrated with buffer T containing 10mM imidazole. After washing the column with 50 mM imidazole in buffer T, His_6-RAD52 was eluted with a 50–500 mM imidazole gradient in T buffer. The fractions were analyzed for presence of RAD52 by SDS-PAGE. Peak fractions were pooled, diluted five times with buffer R (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM DTT, 10% glycerol) and loaded onto a 5-ml HiTrap Heparin HP column (GE Healthcare) equilibrated with buffer R containing 100 mM KCl. After washing with equilibration buffer, RAD52 was eluted with a 0.1–1.0 M KCl gradient in R buffer. Peak fractions were diluted twice with buffer R and aliquots were stored at -70 °C. RPA was overexpressed in bacteria and purified as described previously (Henricksen et al, 1994). RECQ5, RECQ5K58R, and RAD51K133R were overexpressed and purified as described previously (Garcia et al, 2004; Sigurdsson et al, 2002).

Cell culture experiments

HEK293/DR-GFP, HEK293/SA-GFP, U2OS/DR-GFP, U2OS/SA-GFP, and U2OS cells were cultured in Dulbecco's modified Eagle medium (DMEM, OmniLab) supplemented with 10% fetal calf serum (FCS, Gibco) and 100 U/ml penicillin/streptomycin.

siRNA and DNA oligonucleotides

Unless indicated otherwise, all the siRNAs and DNA oligonucleotides (PAGE purified) used in the study were purchased from Microsynth. siBLM_2 was a smartpool siRNA from Dharmacon (a kind gift from Dr. Pietro Pichieri). The sense strand sequences of all siRNAs used are the following:

siLuc: 5'-CGU ACG CGG AAU ACU UCG A dTdT-3'

siRAD51: 5'-AAG GGA AUU AGU GAA GCC AAA dTdT-3'

siRECQ5_1: 5'-CAG GAG GCU GAU AAA GGG UUA dTdT-3'

siRECQ5_2: 5'-GGA GAG UGC GAC CAU GGC U dTdT-3'

siRAD52: 5'-AAG GAU GGU UCA UAU CAU GAA dTdT-3'

siFBH1: 5'-GAU ACA GAG UGA AGA AUG U dTdT-3' (Fugger et al, 2009)

siBLM_1: 5'-CCG AAU CUC AAU GUA CAU AGA dTdT-3'

The sequences of DNA oligonucleotides used are the following:
f9 (59mer): 5'-ACT ATC ATT CAG TCA TGT AAC CTA GTC AAT CTG CGA GCT CGA ATT CAC TGG AGT GAC CT-3'
f7 (30mer): 5'-ATT GAC TAG GTT ACA TGA CTG AAT GAT AGT-3'
f9-C (59mer): 5'-AGG TCA CTC CAG TGA ATT CGA GCT CGC AGA TTG ACT AGG TTA CAT GAC TGA ATG ATA GT -3'

DNA strand-annealing assays

Reactions were carried out in buffer R [20 mM Tris-acetate (pH 7.9), 50 mM Potassium acetate, 10 mM Magnesium acetate, 1mM dithiothreitol] supplemented with ATP-regenerating system (10 U/ml creatine phosphokinase and 12 mM phosphocreatine). In tube A, a 5'-end radiolabeled 59-mer oligonucleotide (f9) at a concentration of 5 nM was preincubated at 30 °C for 3 min with or without 400 nM RAD51K133R in a final volume of 20 µl. In tube B, 5 nM 30-mer oligonucleotide (f7), complementary to 5'-end of f9, was mixed with 120 nM RAD52 and 40 nM RPA in a final volume of 20 µl. Where required, the reaction mixture in tube B also contained 5 nM 59-mer DNA duplex prepared by annealing of unlabeled f9 and f9-C oligonucleotides. The two reactions were mixed together, and where required RECQ5 or RECQ5K58R were added to a final concentration of 80 nM. 5 µl aliquots were removed at the indicated time points and mixed with 2.5 µl of stop solution [125 nM f9 (unlabeled), 33% (v/v) glycerol, 1% (w/v) SDS, 0.15 M EDTA, 0.5 mg/ml proteinase K and 0.1% (w/v) Bromophenol Blue] followed by a 5 min incubation at 30 °C. DNA products were resolved by electrophoresis in 10% PAGE run in 0.5 x TBE buffer, at 100 V for 2 h. Radiolabeled DNA species were visualized by phosphorimaging and quantified using ImageQuant TL software.

DSB repair reporter assays

HEK293/DR-GFP, U2OS/DR-GFP or HEK293/SA-GFP cells, were seeded in a 6-well plate (poly-lysine coated; Sigma Aldrich) at 0.6 x 10^6 cells per well, and transfected 24 h later with appropriate siRNA (40 nM) using RNAiMax according to the manufacturer's instructions (Invitrogen). After 24 h, 0.2 x 10^6 cells for each siRNA tested were plated in a

12 well plate (poly-lysine coated). A day later cells were transfected with 0.6 μg of the I-SceI-expressing plasmid pCBASce (Richardson et al, 1998) or empty vector (pcDNA3.1) using JETprime according to the manufacturer's instructions (Polyplus) followed by a transfection with another dose of siRNA (15 nM) after 6 h. After 48 h, cells were analyzed by flow cytometry on a Cyan ADP (Dako) using Summit software (Beckman Coulter). Maintenance of the DR-GFP and SA-GFP HEK293 cell lines, culture conditions and FACS analysis were done as described previously (Bennardo et al, 2008). For U2OS DR-GFP cells, the above protocol was modified according to (Gunn & Stark, 2012).

Cell cycle analysis

Cells in the DR-GFP and SA-GFP reporter experiments were also subjected to measurement of DNA content. On the day of FACS analysis for GFP positive cells, approximately 0.1 x 10^6 cells were collected for each siRNA treatment, centrifuged and washed twice with PBS, permeablized with 70% ethanol, treated with 0.5 mg/ml RNaseA for 30 min at 37 $^{\circ}$C and finally stained with 50 mg/ml propidium iodide (Sigma Aldrich). The distribution of cell cycle phases with different DNA content was determined using Cyan ADP (Dako) and summit software (Beckman Coulter).

Quantitative real-time PCR

For quantitative real-time PCR (qPCR), RNA was isolated from DR-GFP cells treated either with siLuc or siFBH1 using NucleoSpin RNA II kit (Macherey-Nagel). Total RNA (1.5 μg) was used for cDNA synthesis with Superscript Reverse Transcriptase III (Fermentas). The resulting cDNA served as a template for quantitative PCR performed with a Light Cycler 480 using the SYBR Green I Master Kit (Roche). Absolute values of FBH1 expression were normalized to RPLP0 expression and fold change in siFBH1 sample versus siLuc was calculated using Pfaffl's method. Primers and qPCR conditions used are the following: Tm 60°C, 40 cycles.

RPLP0 (Forward): 5′- CCA GTC TGG AGA AAC TGC TG -3′

RPLP0 (Reverse): 5′-CAG CAG CTG GCA CCT TAT TGG-3′

FBH1 (Forward): 5'- ACA GAG CAC GCT GAA ATG AGA-3'

FBH1 (Reverse): 5'- CCA TCT TTG ACC GAA GGG CT-3'

Whole cell extract preparation and Western blot analysis

Cell pellets were re-suspended in appropriate volume of extraction buffer [50 mM Tris-HCl pH 7.5, 120 mM NaCl, 0.5% (v/v) NP-40, 20 mM NaF, 15 mM sodium pyrophosphate, 1 mM EDTA, 6 mM EGTA] supplemented with 0.1 mM PMSF and 1 x complete protease inhibitor cocktail (EDTA free, Roche). After incubation on ice for 10 min, the cell suspension was sonicated for 5 min at high power, 30" On/Off pulse in Bioruptor (Diagenode) followed by centrifugation at 13,000 rpm for 30 min. Proteins were estimated using Bradford's method.

To check for depletion of proteins, cell extracts (50 μg) were subjected to electrophoresis on either 8% or 12% denaturing SDS-polyacrylamide gel. The proteins were transferred onto PVDF membranes (Hybond, GE Healthcare) in a wet transfer set up at 30V for 16 h. The membranes were blocked in 5% low fat milk in TBST (20 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% Tween 20) and then incubated for 3 h at RT in appropriate primary antibody dilutions in TBST (plus 5% low fat milk). The membranes were then washed thrice in TBST and incubated for 1 h in appropriate secondary HRP-conjugated IgG dilutions in TBST (plus 5% low fat milk). Immune complexes were visualized using ECL detection reagents (Pierce).

SCE analysis

The SCE assay was done as described previously with minor modifications (Bayani & Squire, 2005). Briefly, cells were seeded in a 6-cm plate, and two rounds of transfection with appropriate siRNAs were performed at 24 h and 48 h post seeding. Cells were then grown for 40 h in the presence of 100 μM 5-bromo-2'-deoxyuridine (BrdU), and further incubated for 2 h with 0.2 μg/ml colcemid. Camptothecin (CPT; 40 nM) was added 20 h prior to cell harvest where indicated. Metaphase cells were harvested by mitotic shake-off, swollen in 75 mM KCl for 15 min, fixed with Carnoy's buffer (3:1 methanol and glacial acetic acid), spread on a clean glass slide and air-dried. The slides were then stained with Hoechst 33258 (50 μg/ml) for 30 min, rinsed with PBS, UV (254 nm, Stratalinker) exposed for 10 min and incubated in 2x SSC for 60 min. The slides were finally stained with 7% Giemsa solution for 15 min, washed twice with water and examined under light microscope. All quantifications were carried out blind and 50 metaphases were analyzed from each condition. Statistical analysis was performed using GraphPad Prism software.

Acknowledgements

We thank Dr. Jeremy Stark for the HEK293/DR-GFP, HEK293/SA-GFP and U2OS/DR-GFP cell lines, Dr. Stephen West for providing the BL21 pLysS strain over-expressing human RAD52 protein and Dr. Pietro Pichierri for BLM siRNA. This research study was supported by grants from the Swiss National Science Foundation, Promedica Stiftung, Stiftung zur Krebsbekämpfung and Theodor und Ida Herzog-Egli Stiftung.

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Figure legends

Figure 1. RECQ5 promotes homologous recombination with non-crossover outcome. **(A)** Scheme of the DR-GFP reporter for HR-mediated DSB repair. The site-specific DSB in the reporter cassette is generated by I-SceI endonuclease. Only NCO events give rise to a functional GFP allele. **(B)** Western blot analysis of extracts from HEK293/DR-GFP cells transfected with indicated siRNAs. Blots were probed with indicated antibodies. **(C)** Efficiency of HR-mediated repair of I-SceI-induced DSB in HEK293/DR-GFP cells treated with indicated siRNAs. Cells were transfected with appropriate siRNA (40 nM) two days prior to transfection of I-SceI-expressing plasmid. Percentage of GFP positive cells was measured by flow cytometry two days after DSB induction and taken as a measure of DSB repair efficiency. Values plotted represent relative repair efficiency calculated as a percentage of repair efficiency measured in cells transfected with control siRNA (siLuc; 100%). All data points represent an average of at least three replicates with error bars indicating standard deviation. **(D)** Western blot analysis of extracts from U2OS/DR-GFP cells transfected with indicated siRNAs. Blots were probed with indicated antibodies. **(E)** Relative efficiency of HR-mediated repair of I-SceI-induced DSB in U2OS/DR-GFP cells treated with indicated siRNAs as compared to cells transfected with control siRNA. Experiments were performed as in (C) except that the flow cytometry analysis was performed 3 days after I-SceI transfection. All data points represent an average of at least three replicates and the error bars represent the standard deviation. HR, homologous recombination; DSB, double-strand break; NCO, non-crossover; siRNA, small interfering RNA; GFP, green fluorescent protein.

Figure 2. RECQ5 suppresses inhibitory effect of RAD51 on DNA double-strand break repair by single-strand annealing. **(A)** Scheme of the SA-GFP reporter cassette integrated in HEK293 cells. SSA-mediated repair of I-SceI-generated DSB results in the formation of a functional GFP allele. **(B)** Western blot analysis of extracts from HEK293/DR-GFP cells transfected with indicated siRNAs. The blots were probed with indicated antibodies. **(C)** Efficiency of SSA-mediated repair of I-SceI-induced DSB in HEK293/SA-GFP cells transfected with indicated siRNAs. Cells were transfected with appropriate siRNA (40 nM) two days prior to transfection of I-SceI-expressing plasmid. Percentage of GFP-

positive cells was determined by flow cytometry two days after DSB induction and taken as a measure of repair efficiency. Values plotted represent relative repair efficiency calculated as a percentage of repair efficiency measured in cells transfected with control siRNA (siLuc; 100%). All data points represent an average of at least three replicates with error bars indicating standard deviation. For RECQ5 down-regulation, siRECQ5_2 was used. SSA, single-strand annealing; DSB, double strand break; GFP, green fluorescent protein.

Figure 3. RECQ5 helicase counteracts the inhibitory effect of RAD51 on RAD52-mediated ssDNA annealing *in vitro*. **(A)** *Upper panel*: Reaction scheme depicting the effect of RAD51 (green ovals) on annealing of two complementary oligonucleotides (59-mer and 30-mer represented by red and blue lines, respectively) in presence of RAD52 and RPA. RAD52 is depicted as a heptameric ring structure (red ovals). 30-mer oligonucleotide can accommodate binding of one RPA heterotrimer (light blue ovals). *Lower panel*: All reactions were carried out at 30°C in buffer R supplemented with ATP-regenerating system. Annealing reactions contained 5'-end radiolabeled 59-mer oligonucleotide (2.5 nM), either free or pre-coated with RAD51K133R (200 nM), a 30mer oligonucleotide (2.5 nM) complementary to the 5'-half of the 59-mer, RAD52 (60 nM) and RPA (30 nM). Where indicated, RECQ5 or RECQ5K58R were present at a concentration of 80 nM. Reaction aliquots at indicated time points were subjected to PAGE followed by phosphorimaging as described in Materials and Methods. (**B**) Quantification of data shown in (A). Each data point represents the mean of three independent experiments. Error bars represent standard deviation. **(C)** *Upper panel*: Reaction scheme depicting the effect of RAD51 on annealing of two complementary oligonucleotides in presence of a homologous duplex, RAD52 and RPA. RAD51 filament formed on the radiolabelled oligonucleotide (red line with asterisk) inhibits RAD52/RPA-mediated annealing and promotes strand exchange with the homologous duplex. *Lower panel*: Reactions were carried out and analyzed as in (B). Homologous 59-mer duplex was present at a concentration of 2.5 nM. Schemes of radiolabeled DNA species are shown on left. Radioactive label at the 5'-end is depicted by asterisks. **(D)** Quantification of data shown in (C). Each data point represents the mean of three independent experiments. Error bars represent standard deviation. RPA; Replication protein A; PAGE, polyacrylamide gel electrophoresis.

Figure 4. RECQ5 and BLM act in different pathways to suppress crossovers in human cells. **(A)** Frequency of spontaneous SCEs in U2OS cells transfected with indicated siRNAs. **(B)** Frequency of CPT-induced SCEs in U2OS cells transfected with indicated siRNA. Cells were treated with 40 nM CPT for 20 h where indicated. SCE assay and analysis was conducted as described in Materials and Methods. Each data point represents number of SCEs per chromosome in a single metaphase spread. 50 metaphase spreads were analyzed from each condition. (C) Western blot analysis of extracts from U2OS cells transfected with indicated siRNAs. Blots were probed with indicated antibodies. (D) Model for the roles of RECQ5 and BLM in suppression of COs during DSB repair by HR. RECQ5 promotes SDSA by disrupting aberrant RAD51-filaments formed after unwinding of the extended D-loop. BLM acts as a part of the BTR (BLM-TOPOIIIα-RMI1/2) complex to mediate dissolution of dHJs. RAD51-filaments formed during the post-synaptic phase of SDSA can promote re-invasion of the homologous duplex followed by formation of a dHJ structutre, increasing the risk of COs. HR, homologous recombination; SCE, sister chromatid exchange; CPT, camptothecin; SDSA, synthesis dependent strand annealing; dHJ, double Holliday junction; DSBR, double-strand break repair.

Supplementary information

Supplementary Figure legends

Figure S1. Confirmation of FBH1 knockdown by RT-qPCR. Graphical representation of FBH1 expression level in HEK293/DR-GFP cells treated with indicated siRNA normalized against RPLP0 expression level. mRNA was extracted from HEK293/DR-GFP cells treated with 40 nM siRNA for 2 days and with 15nM siRNA for the next 2 days. After cDNA synthesis, qPCR was performed using primers against the FBH1 gene and the RPLP0 gene (house-keeping control). The Pfaffl equation was used for normalization and calculation of relative FBH1 expression levels. RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.

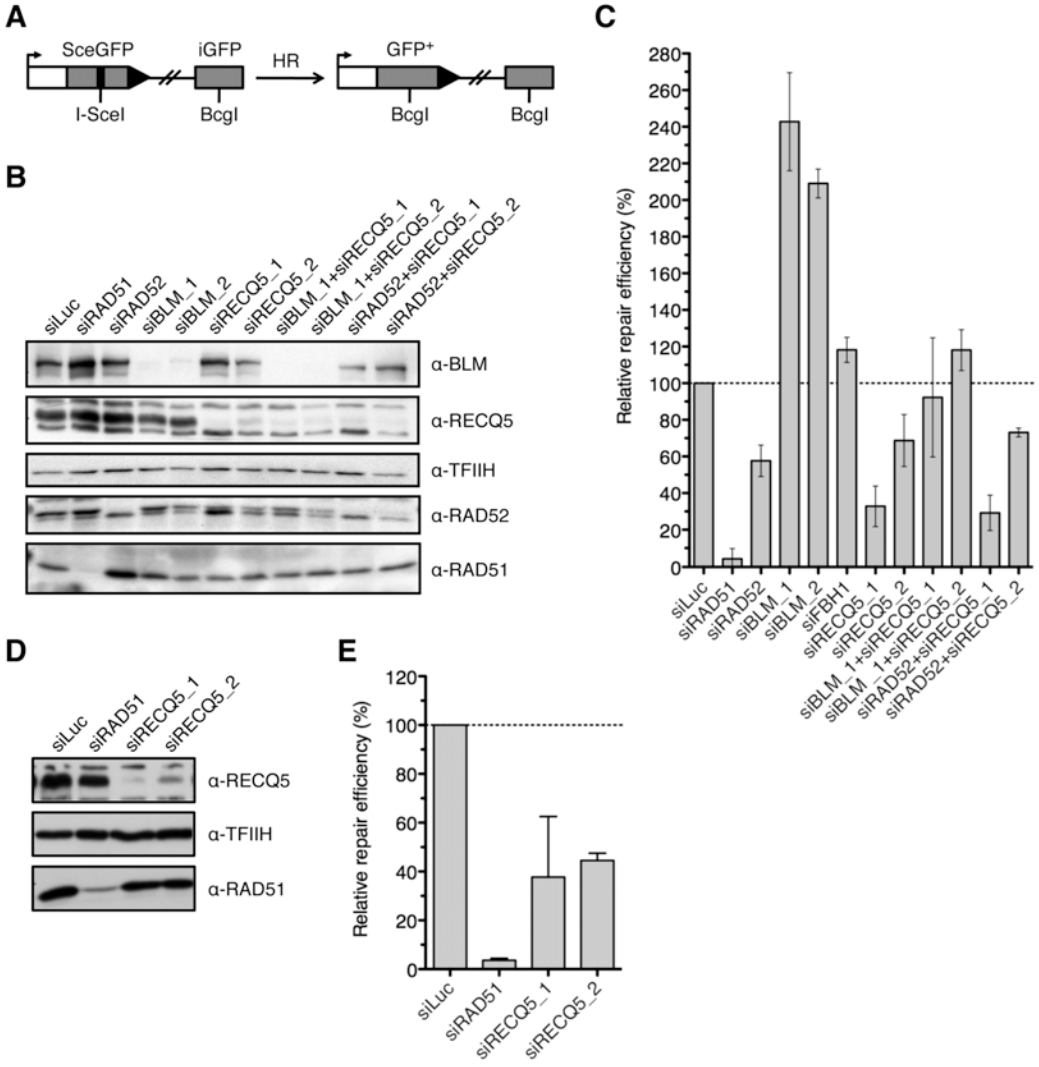
Figure S2. Cell-cycle profiles of HEK293/DR-GFP and HEK293/SA-GFP cells upon depletion of RECQ5. Cells were transfected with indicated siRNAs and I-SceI expression vector under conditions followed in DR-GFP and SA-GFP reporter assays (Figure 1 and 2). After fixation and Propidium Iodide staining, cells were subjected to flow cytometry analysis.

Figure S3. RECQ5 suppresses inhibitory effect of RAD51 on DNA double-strand break repair by single-strand annealing. HEK293/Sa-GFP cells were transfected with either siLuc, siRAD51, siRECQ5_1 or both siRAD51 and siRECQ5_1 followed by transfection of I-SceI-expressing plasmid as described in Materials and methods. Percentage of GFP-positive cells was measured by flow cytometry two days after DSB induction and taken as a measure of DSB repair efficiency. Values plotted represent relative repair efficiency calculated as a percentage of repair efficiency measured in cells transfected with control siRNA (siLuc; 100%). All data points represent an average of at least three replicates with error bars indicating standard deviation. SSA, single-strand annealing; DSB, double-strand break; GFP, green fluorescent protein.

Figure S4. RAD52-mediated DNA annealing. The two complementary oligonucleotides, 5'-end radiolabeled 59mer (f9) and 30mer (f7), at a concentration of 2.5 nM were

incubated for 5 minutes in presence of RPA (30 nM) and homologous 59mer duplex (2.5 nM). This was followed by addition of RAD52 (60 nM) where indicated. Reaction aliquots were collected at indicated time points and analyzed as in Figure 3.

Figure 1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α-BLM
α-RECQ5
α-TFIIH
α-RAD52
α-RAD51
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Relative repair efficiency (%)
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siRAD51
siRAD52
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siFBH1
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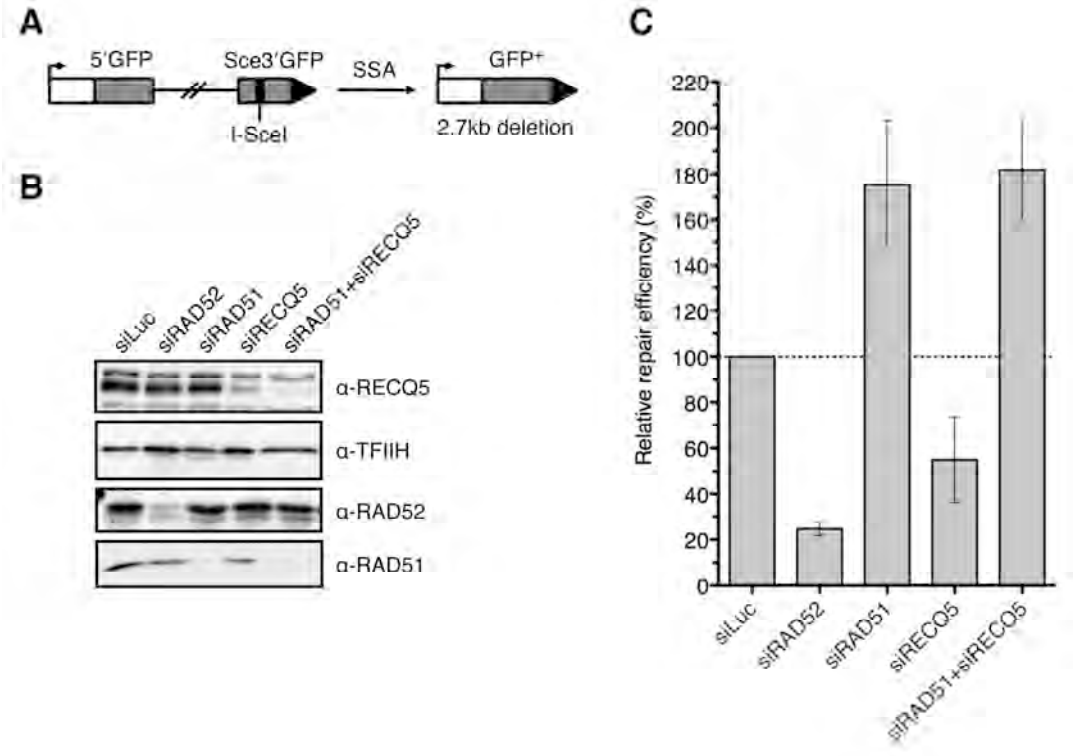
siRECQ5_2

Figure 2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SSA
GFP+
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α-RECQ5
α-TFIIH
α-RAD52
α-RAD51
C
Relative repair efficiency (%)
220
200
180
160
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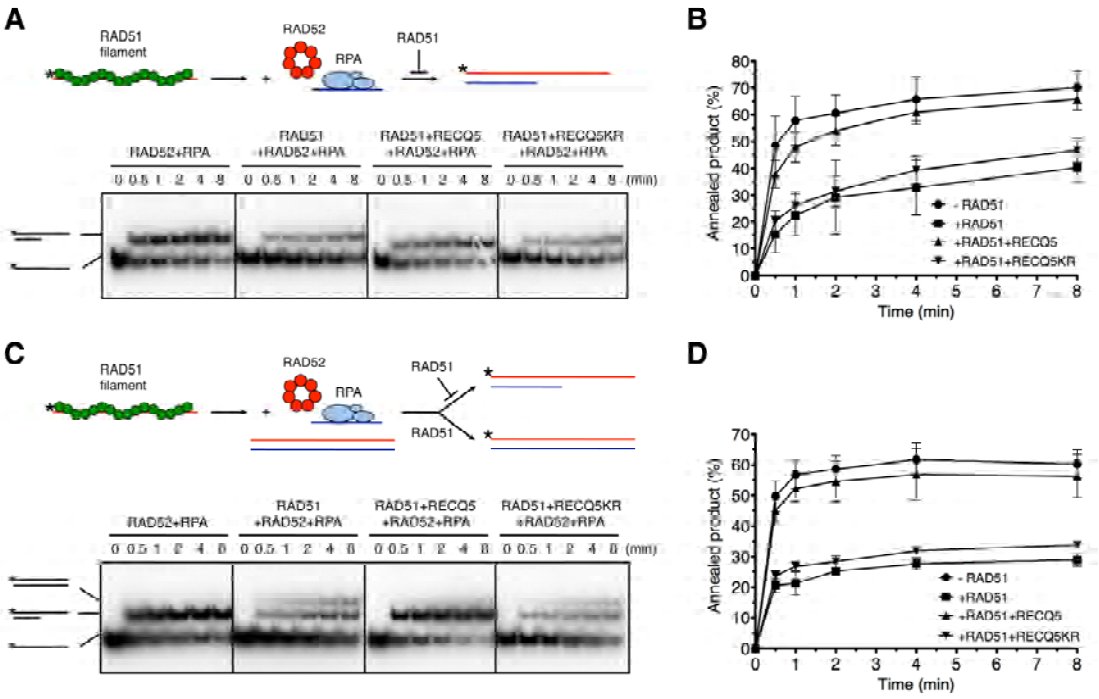
Figure 3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0 0.5 1 2 4 8 (min)
B
Annealed product (%)
Time (min)
-RAD51
+RAD51
+RAD51+RECQ5
+RAD51+RECQ5KR
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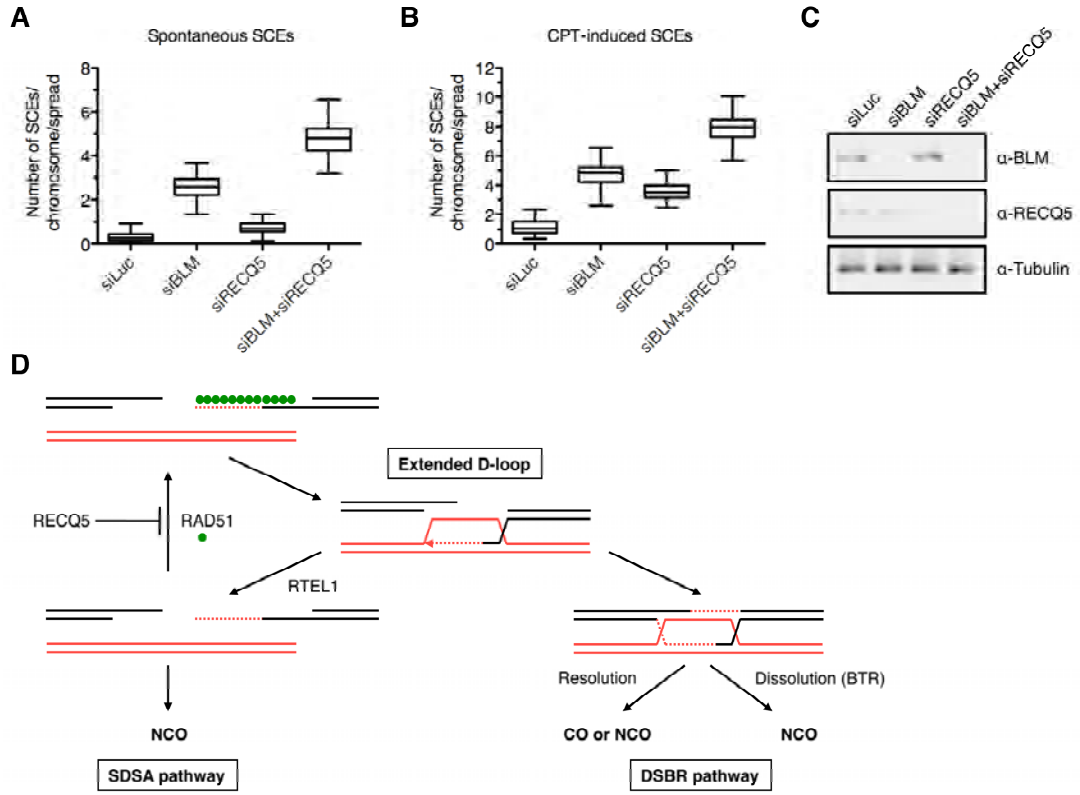
Figure 4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siBLM+siRECQ5
C
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siBLM
siRECQ5
siBLM+siRECQ5
α-BLM
α-RECQ5
α-Tubulin
D
Extended D-loop
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RTEL1
Resolution
Dissolution (BTR)
NCO
CO or NCO
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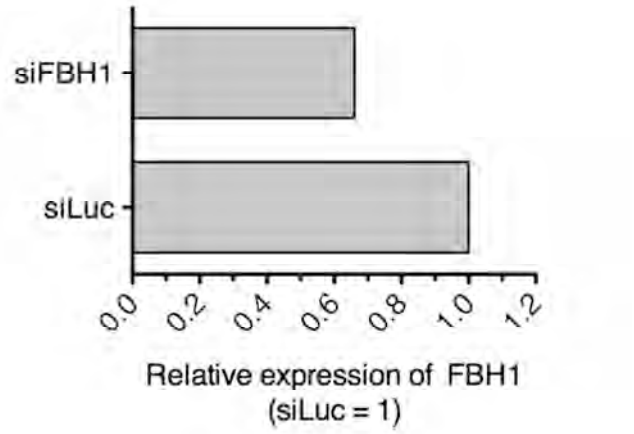
SDSA pathway
DSBR pathway

Figure S1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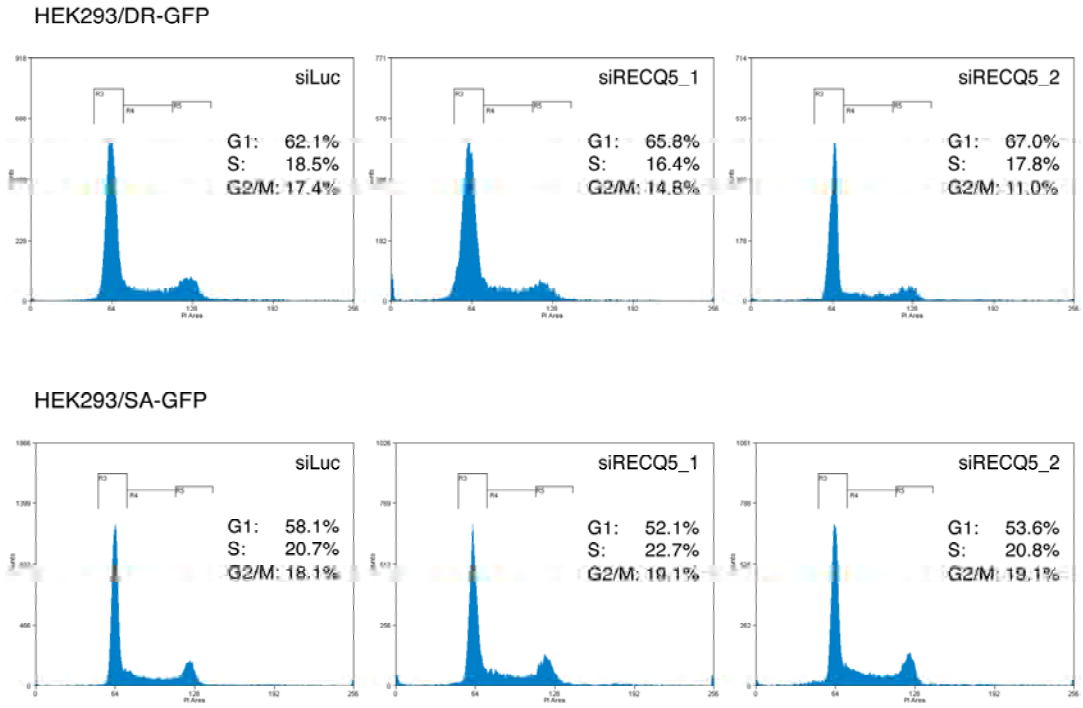
1.0
1.2
Relative expression of FBH1
(siLuc = 1)

Figure S2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S: 18.5%
G2/M: 17.4%
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G2/M: 14.8%
siRECQ5_2
G1: 67.0%
S: 17.8%
G2/M: 11.0%
HEK293/SA-GFP
siLuc
G1: 58.1%
S: 20.7%
G2/M: 18.1%
siRECQ5_1
G1: 52.1%
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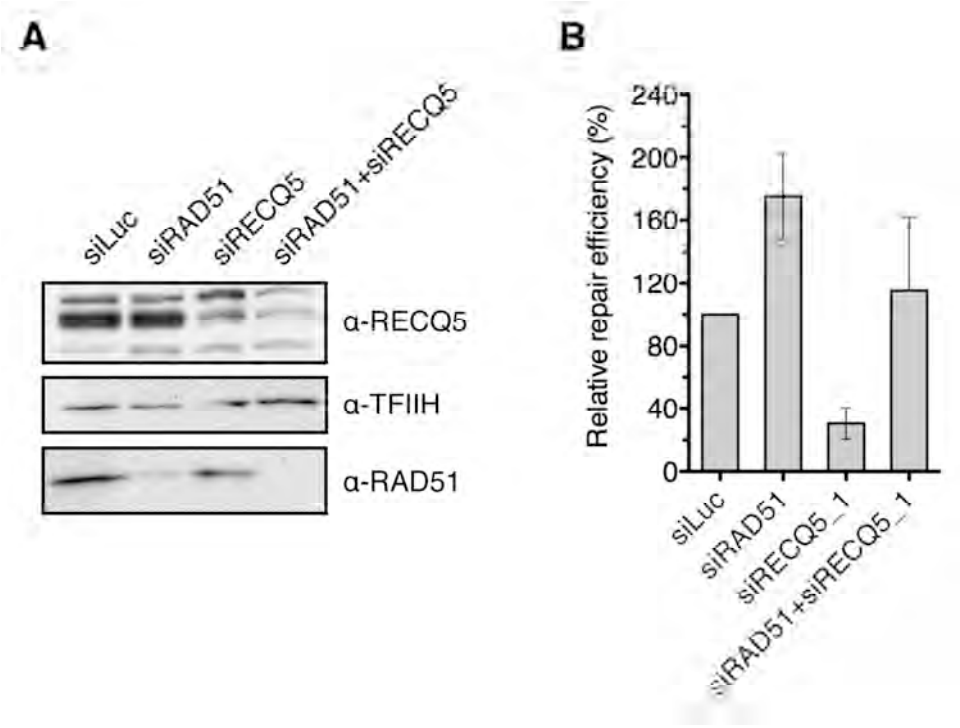
Figure S3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α-RAD51
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Relative repair efficiency (%)
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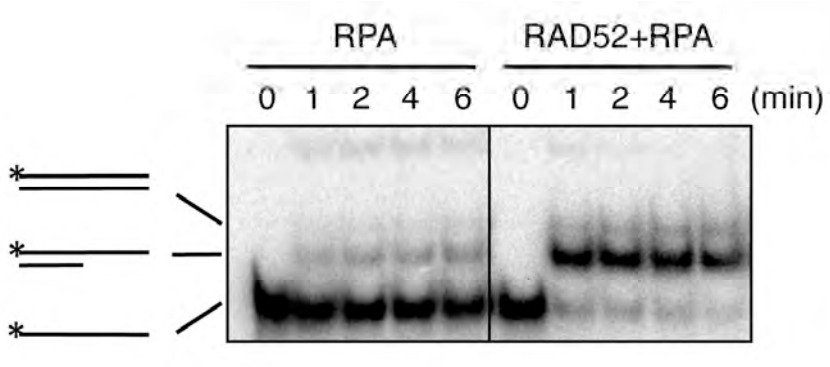
200
160
120
80
40
0
siLuc
siRAD51
siRECQ5_1
siRAD51+siRECQ5_1

Figure S4



4 DISCUSSION

Since the cloning of RECQ5 gene, 15 years ago, a varying spectrum of biochemical functions and cellular roles have been assigned to RECQ5 (Kitao et al, 1998). All of these functions somehow connect RECQ5 to DNA metabolic activities including replication, recombination, repair and transcription, resulting ultimately to maintenance of genomic stability (Aygun & Svejstrup, 2010). MS-based analyses have revealed that the interactome of RECQ5 is quite diverse but the purpose for many interactions is still elusive (Izumikawa et al, 2008; Zheng et al, 2009). This study was aimed at comprehensive characterization of the RECQ5-RAD51 interaction and its biological significance.

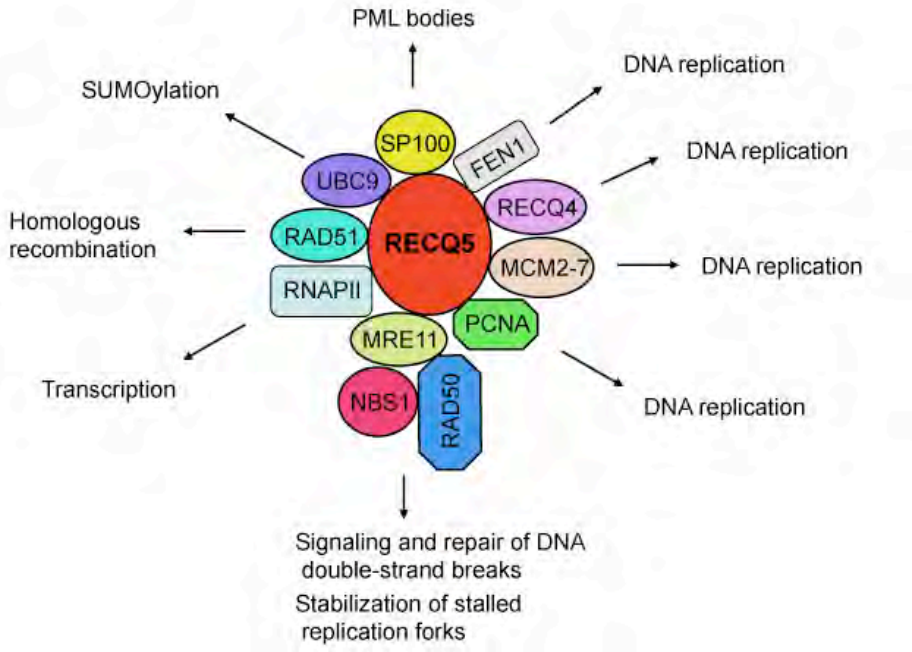


Figure 8: **Brief overview of RECQ5 interactome.** RECQ5 interacts with various proteins that link it with DNA replication, DNA repair, and transcription.

4.1 Molecular mechanism underlying disruption of RAD51-ssDNA filaments by RECQ5

RAD51 is the central player in the process of HR. As described in the Introduction section, RAD51 forms a helical filament on ssDNA, along with other mediator proteins, that is responsible for search of the homologous DNA duplex and for strand invasion resulting in the formation of a D-loop. It has been shown that RECQ5 physically interacts with RAD51 and inhibits RAD51-mediated D-loop formation *in vitro* (Hu et al, 2007). Furthermore, it has been demonstrated that RECQ5 displaces RAD51 from ssDNA *in vitro* and that its deficiency results in genomic instability (Hu et al, 2007). This implies that RECQ5 functions in HR and possibly exerts its function through disruption of recombinogenic RAD51-ssDNA filaments.

In the first part of the study, our central question was the molecular mechanism underlying disruption of RAD51-ssDNA filaments by RECQ5. For this, we initially characterized the RECQ5-RAD51 interaction. Pull down assays helped us map the RAD51 interaction domain of RECQ5 to an 18 amino-acid region (652-674) and show that the F666 residue is critical for RECQ5-RAD51 interaction. Using inducible U2OS-TREx cell lines harboring a stably integrated vector for expression of FLAG-tagged RECQ5 WT or its F666A mutant (FA), we investigated the role of RECQ5-RAD51 interaction in RECQ5 recruitment to sites of laser-induced DNA damage (laser lines). This analysis indicated that RECQ5 is recruited to DSBs in a manner dependent on its interaction with RAD51. However, although the FA mutant was highly compromised in its recruitment to RAD51 positive laser lines, it was still recruited to laser lines that did not have any RAD51, suggesting that RECQ5 might also have a role in a RAD51-independent DSB repair pathway.

Our studies have shown that RECQ5-RAD51 interaction partially contributes to the filament disruption acivity of RECQ5 (Schwendener et al, 2010). These data suggest that RECQ5 displaces RAD51 from ssDNA filaments by direct protein-protein interaction and its ssDNA-translocase activity probably drives its movement from one RAD51 molecule to the next. The helicase activity of RECQ5 is dispensable for its ability to disrupt RAD51-ssDNA filaments. A similar mechanism has been proposed for the yeast DNA helicase Srs2 that also interacts directly with Rad51 and promotes disassembly of

Rad51-ssDNA filaments (Colavito et al, 2009; Krejci et al, 2004; Krejci et al, 2003). Interestingly, the 1-475 deletion variant of RECQ5 showed a highly compromised filament disruption activity compared to the 1-651 mutant, suggesting that there is an additional domain between the helicase and RAD51-interacting domain of RECQ5 that contributes to this activity. To address this hypothesis, we tested a series of internal deletion variants of RECQ5 (Δ461-475, Δ515-568, Δ543-607, Δ569-594 and Δ571-653). We observed that the variants Δ515-568, Δ543-607, Δ569-594 could not promote efficient filament disruption, whereas the variants Δ461-475 and Δ571-653 were as active as the full-length RECQ5. This suggests that the region between aa 465-570 of RECQ5 harbors a second domain involved in RAD51-ssDNA filament disruption.

Srs2 seems to disrupt RAD51-ssDNA filaments by destabilizing RAD51 assembly on ssDNA by stimulating hydrolysis of RAD51-bound ATP mediated by direct protein-protein interaction (Antony et al, 2009). As RECQ5 can disrupt filaments made by the RAD51K133R mutant that is defective for ATP hydrolysis, it seems that RECQ5 differs mechanistically from Srs2. Our data suggests that RECQ5 is recruited to the RAD51 filament *via* its RAD51 interaction domain and then it translocates along the ssDNA. It is possible that during translocation, the domain of RECQ5 spanning aa 465-570 forms a wedge-like structure ahead of RECQ5 that dislodges RAD51 from the ssDNA. This model justifies the requirement of the two domains of RECQ5 in disruption of RAD51-ssDNA filaments.

4.2 Role of RECQ5 in promotion of DSB repair by HR

The second part of this PhD study dealt with the cellular relevance of the RAD51-ssDNA filament disruption activity of RECQ5. To answer this question, we first analyzed the effect of RECQ5 depletion on DSB repair by HR in human cells harboring a GFP-based reporter cassette, DR-GFP, detecting selectively NCO events (Bennardo et al, 2008). Recent evidence suggests that SDSA is the preferred pathway of HR during mitosis in both yeast and mammalian cells implying that the DR-GFP reporter primarily scores for repair *via* SDSA (Matos et al, 2011; Mitchel et al, 2010). We found that RECQ5 is required for formation of NCO products in a cell line-independent manner. The two different siRNA sequences against RECQ5 did not result in equivalent levels of RECQ5 depletion from the

cells, as detected by western blotting, and this may explain their differential effect on DSB repair efficiency in the DR-GFP system. In contrast to RECQ5, depletion of BLM resulted in a dramatic increase in repair efficiency, pointing to a new role of BLM during HR, separate from its role in dissolution. It is possible that BLM disrupts annealed structures during SDSA or unwinds the D-loop prior to DNA synthesis (Bachrati et al, 2006; Sikdar et al, 2009).

Though most prevalent in mitotic cells, the SDSA sub-pathway of HR is poorly characterized. SSA is another DSB repair pathway that is initiated by resection of the DSB ends followed by annealing of complementary DNA sequences and restoration of the continuity of DNA strands. Since SDSA is mechanistically similar to SSA, we utilized a human cell line harboring an SSA reporter cassette, SA-GFP, to investigate the possible role of RECQ5 in annealing of complementary DNA strands. As reported for yeast, we observed that depletion of RAD51 in human cells led to a striking increase in SSA repair efficiency compared to control cells, indicating that RAD51 has inhibitory effect on SSA (Lee & Lee, 2007; Stark et al, 2004). Depletion of RECQ5 from SA-GFP cells negatively affected the repair efficiency. Surprisingly, depletion of RECQ5 from RAD51-depleted cells did not have any effect on the level of repair by SSA compared to RAD51-depleted cells suggesting that RECQ5 is required for SSA only in the presence of RAD51. Initially, it was considered that the effect of RAD51 on SSA repair is indirect as its absence may channel repair of the resected DSB lesion towards SSA pathway but our observation with co-depletion of RECQ5 and RAD51 provides evidence that RAD51 has a direct effect on repair by SSA. To conclude, RAD51 inhibits SSA and RECQ5 is required to relieve this inhibitory effect of RAD51. As afore-mentioned, SSA and SDSA are mechanistically similar so these observations can be extrapolated to the SDSA pathway.

It has been shown that a DNA sequence coated by yeast Rad51 cannot be annealed to the complementary DNA strand even in the presence of Rad52 and RPA (Wu et al, 2008). Observations from the reporter cells led us to conceive the possibility that the presence of RAD51, after D-loop disruption, may promote re-formation of 'illegitimate' RAD51-ssDNA filaments. This in turn could promote re-invasion of the homologous duplex and channel the recombination intermediate towards the DSBR sub-pathway of HR. Such illegitimate RAD51-ssDNA filaments could be disrupted by RECQ5, thus establishing the need for RECQ5 during SDSA. This hypothesis was validated by results

from our biochemical experiments where we analyzed the effect of RAD51, in absence and presence of RECQ5, on annealing of two complementary DNA oligonucleotides in the presence of RAD52 and RPA. Taken together, we could show that human RAD51 inhibits RAD52-mediated annealing of complementary DNA sequences and RECQ5 promotes annealing by counteracting this inhibitory effect.

To prevent COs in a mitotic cell, dHJ structures are processed by dissolution mediated by BLM, BLAP75, and TOPO IIIα (Matos et al, 2011). In the absence of BLM, such structures are resolved by resolvases to give preferentially a CO product (Mitchel et al, 2010). This is further supported by the observation that BLM depletion leads to elevated levels of SCEs (Hu et al, 2005; Wang et al, 2003). Coupled with our hypothesis that RECQ5 promotes SDSA, these observations suggest that depletion of RECQ5 in BLM deficient cells would lead to an increase in CO product formation. Indeed, SCE analysis in BLM and RECQ5 co-depleted cells showed much higher levels of SCEs compared to BLM depletion alone, thus further validating our model where RECQ5 is needed to promote SDSA pathway, possibly by disrupting illegitimate post-synaptic RAD51-ssDNA filaments.

Evidence for this model can also be drawn from studies in yeast. The Srs2 DNA helicase in yeast has been shown to disrupt RAD51-ssDNA filaments through direct interaction with RAD51 (Colavito et al, 2009; Krejci et al, 2003). Cells deficient for Srs2 demonstrate a defect in carrying out gene conversion leading to NCO products. This gets reflected in a drop in overall repair efficiency, increasing the proportion of CO events (Ira et al, 2003). Moreover, although the Srs2 DNA helicase is capable of unwinding synthetic D-loops and its helicase activity is stimulate by RAD51 filament on dsDNA, it cannot disrupt RAD51-made D-loops (Dupaigne et al, 2008; Prakash et al, 2009). Taken together, these observations suggest that Srs2 promotes SDSA by regulating RAD51 filament stability on post-synaptic recombination intermediates rather than by disrupting D-loops. Also, Srs2 has been shown to promote annealing in the SSA pathway (Sugawara et al, 2000; Wilson, 2002).

There is no obvious homolog of Srs2 in humans and the search for a functional ortholog has resulted in many potential candidates such as BLM, PARI, FBH1 (Bugreev et al, 2007; Fugger et al, 2009; Moldovan et al, 2011). Our results suggest that RECQ5 perfectly fits the criteria to be a functional orthologue of Srs2. Both of them interact with

RAD51 and disrupt RAD51-ssDNA filaments. Furthermore, both of them promote formation of NCO products by SDSA, probably by disrupting illegitimate post-synaptic RAD51-ssDNA filaments.

5 CONCLUSIONS AND FUTURE PERSPECTIVE

Repair of DSBs by HR is crucial for maintenance of genomic integrity by preventing detrimental deletions/insertions or chromosomal rearrangements, which can induce tumorigenesis. Textbook knowledge of HR is largely restricted to the canonical DSBR pathway. However, recent evidence shows that SDSA is the dominant HR sub-pathway for DSB repair in mitotic cells (Matos et al, 2011; Mitchel et al, 2010). The current state of knowledge about the molecular mechanism of SDSA is extremely poor. Although the steps leading to formation of a NCO product during SDSA are well understood, the information about players and regulators involved at each step is still missing.

Most of our current understanding of this pathway comes from studies in yeast where it has been shown that after D-loop disruption by helicases, such as Mph1, the two DNA ends are annealed by Rad52 in a reaction facilitated by RPA (Prakash et al, 2009; Wu et al, 2008). The DNA helicase Srs2 has an important function in regulating HR by disrupting illegitimate RAD51-ssDNA filaments (Krejci et al, 2003). Evidence suggests that Srs2 promotes repair by SDSA pathway, probably by acting on post-synaptic RAD51-ssDNA filaments.

In this study, we propose that RECQ5 is the most potential Srs2 ortholog in humans. The project was initiated with the aim of elucidating the molecular mechanism and the physiological relevance of RECQ5's ability to disrupt RAD51-ssDNA filaments. We could map the RAD51 interaction domain of RECQ5. Moreover, we could demonstrate that two domains of RECQ5, the RAD51 interaction domain and the region between aa 476-570, are required for catalyzing efficient RAD51-ssDNA filament disruption. Furthermore, we could show that RAD51 inhibits annealing of complementary DNA sequences and RECQ5 counteracts this inhibitory effect. This explains the requirements of RECQ5 for NCO product formation by SDSA pathway as in absence of RECQ5 aberrant RAD51-ssDNA filaments impede SDSA.

However, the question of how RECQ5 distinguishes between appropriate (presynaptic) and inappropriate (post-synaptic or illegitimate) RAD51-ssDNA filaments is a matter of further investigation. It is known that mediator proteins such as BRCA2 and RAD51 paralogs play an important role in stabilizing presynaptic RAD51-ssDNA

filaments so examination of their regulation during HR might shed some light on the problem. Furthermore, it is known, for example, for Srs2 that Cdk1-mediated phosphorylation activates it for promoting SDSA (Saponaro et al, 2010). It will be interesting to investigate if a similar regulatory mechanism is operational in case of RECQ5.

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7 APPENDIX

7.1 The ubiquitin-selective segregase VCP/p97 orchestrates the response to DNA double-strand breaks

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I contributed to this publication by performing the DR-GFP and NHEJ-GFP (EJ5) reporter assays in HEK293 cells, resulting in Figure 2D.

7.2 Prolyl Isomerase PIN1 Regulates DNA Double-Strand Break Repair by Counteracting DNA End Resection

Martin Steger, Olga Murina, Daniela Hühn, Reto Walser, Kay Hänggi, Lorenzo Lafranchi, Christine Neugebauer, **Shreya Paliwal,** Pavel Janscak, Bertran Gerrits, Oliver Zerbe and Alessandro A. Sartori

Manuscript under revision

I contributed to this publication by performing the DR-GFP and NHEJ-GFP (EJ5) reporter assays in HEK293 and U2OS cells.

8 Curriculum Vitae

Surname: **PALIWAL**

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Nationality: Indian

Education and Research Experience

2000 - 2002	All India Senior Secondary Certificate Examination (High-School) Holy Child School, Delhi, India
2002 - 2005	***Bachelor of Science (Hons.) in Biochemistry*** University Of Delhi, India Major: Biochemistry
2004 - 2005	***Summer internship*** in the group of Dr. Pradeep Burma Dept. of Genetics, University of Delhi, India Practical training in recombinant DNA technology
2005 - 2007	***Master of Science in Plant Molecular Biology and Biotechnology (Hons.)***, University Of Delhi, India Major: Molecular Biology, Biotechnology
2005 - 2006	***Summer internship*** in the group of Dr. Shaila Srinivasan Piramal Life Sciences, Mumbai, India Got introduced to high throughput technology for drug screening
2006 - 2007	***Master project*** (one year) in the group of Dr. Sanjay Kapoor Dept. of Plant Molecular Biology and Biotechnology University of Delhi, India **Project title:** Genome wide analysis of rice ribosomal protein genes under different temporal stages of reproduction and under conditions of stress
2007 - 2008	***Research Assistant*** in the group of Prof. Dr. V. Nagaraja Indian Institute of Science, Bangalore, India

	Worked on biochemical characterization of a mycobacterium DNA repair protein, Mfd
2008 - Present	***Ph.D. in Cancer Biology,*** IMCR (Dr. Pavel Janscak) University of Zurich, Switzerland **Project title:** Role of RECQ5 DNA helicase in DNA double-strand break repair by Homologous recombination

List of Publications

Schwendener S, Raynard S, Paliwal S, Cheng A, Kanagaraj R, Shevelev I, Stark JM, Sung P, and Janscak P. **J Biol Chem.** 2010

Meerang M, Ritz D, Paliwal S, Bosshard M, Vitanescu M, Janscak P, Hubscher U, Meyer M and Ramadan K. **Nature Cell Biol**. 2011

Paliwal S, Kanagaraj R, Janscak P. **Manuscript in preparation.**

Steger M, Murina O, Huehn D, Walser R, Haenggi K, Lafranchi L, Neugebauer C, Paliwal S, Janscak P, Gerrits B, Zerbe O and Sartori AA. **Manuscript under revision**.

Prizes, Awards and Fellowships

Cancer Biology PhD Program **Travel award in 2012** to attend the Abcam's conference on Maintenance of Genome Stability

Awarded the **CSIR fellowship in 2008** for pursuing doctoral studies in India

Monsato **Merit Scholarship in 2006 - 2007** for securing third rank in the university examination

Teaching and Mentoring Experience

2011 - 2012	Supervision of a Master student in the group of Dr. Pavel Janscak at the University of Zurich
2011 and 2012	Teaching Assistant for BIO246, Genomic Instability and Molecular Cancer Research, 3 weeks, full time practical block course for Master students at the University of Zurich

Conferences and Presentations

Abcam's conference on Maintenance of Genome Stability, March 2012, Nassau, Bahamas, "Role of RECQ5 DNA helicase in regulation of DNA double strand break repair", *Poster presentation*; Paliwal S, Janscak P.

Replication, recombination and repair club, Cancer Network Zurich, Switzerland, January 2012, "Role of RECQ5 DNA helicase in regulation of DNA double strand break repair", *oral presentation*; Paliwal S, Janscak P.

Cancer Biology PhD Program Retreat, October 2010, Switzerland, "Role of RECQ5 in suppression of Homologous recombination", *poster presentation*; Paliwal S, Radhakrishnan K, Janscak P.

Cancer Network Retreat, October 2009, Ascona, Switzerland, "Elucidating the role of human RECQ5 protein in DNA recombination and repair", *poster presentation*; Paliwal S, Schwendener S, Raynard S, Janscak P.

Extra-curricular activities

Travelling and Hiking

Doing Creative projects like street photography

Volunteering for ASHA (http://www.asha-zurich.ch/) We organize fund-raisers in Zürich to create awareness and raise money for education of under-priviliged children in India

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